IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE HONORABLE BOARD OF PATENT APPEALS AND
INTERFERENCES

In re the Application of Bach *et al.*
Application No.: 08/986,568
Filed: December 5, 1997
Docket No.: 040388/0110
For: **METHOD FOR TREATING ESTABLISHED SPONTANEOUS AUTO-IMMUNE DISEASES IN MAMMALS**

BRIEF ON APPEAL

Appeal from Group 1644

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I. REAL PARTY IN INTEREST

The real party in interest is Diabetogen Biosciences Inc., the exclusive licensee of the present application, which is assigned to I.N.S.E.R.M. by virtue of the assignment recorded in the U.S. Patent and Trademark Office on April 3, 1998.

II. RELATED APPEALS AND INTERFERENCES

Appellant, appellant's legal representative, and the assignee of the present application are not aware of any other appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF CLAIMS

Claims 1, 2, 4-7, 9-13, and 16-18 are pending and are appealed herein. Claims 3, 8, 14 and 15 were canceled. A copy of appellant's pending claims is attached hereto as APPENDIX A.

IV. STATUS OF AMENDMENTS

No amendments have been filed subsequent to the Final Action.

V. SUMMARY OF THE INVENTION

Appellants' claimed invention relates to treating spontaneous and ongoing autoimmune disease by administering a therapeutically effective amount of a non-mitogenic, anti-CD3- active compound to a human (specification, page 3, lines 26-32). By this treatment one can achieve a durable remission of established disease, through the induction of antigen-specific unresponsiveness (specification, page 2, last paragraph continuing to page 3, line 10).

VI. ISSUES ON APPEAL

The issues in this appeal are:

(1) Whether the examiner properly reopened prosecution of the instant case following a Board decision announcing a new lack-of-novelty rejection under 37 C.F.R. §1.196(b).

(2) Whether claims 1, 6, 9-13 and 17-18 are described in the specification in such a way as to reasonably convey, under 35 U.S.C. § 112, 1st ¶, to one skilled in the art that the inventors possessed the claimed invention at the time the application was filed.

(3) Whether the specification enables, under 35 U.S.C. § 112, 1st ¶, one of skill in the art to practice the inventions of claims 1, 2, 4-7, 9-13 and 16-18 without undue experimentation.

(4) Whether claims 1-2, 4-6, 9-13 and 16-18 are obvious, under 35 U.S.C. § 103(a), over Chatenoud *et al.*

VII. GROUPING OF CLAIMS

The appealed claims stand or fall together.

VIII. SUMMARY OF THE ARGUMENT

The examining core arbitrarily and capriciously reopened prosecution of the application following a new lack-of-novelty rejection issued by the Board under 37 C.F.R. §1.196(b). Furthermore, levying new rejections after five years of prosecution and an appeal to the Board epitomizes piecemeal examination. USPTO rules, therefore, compel a reversal of the examiner's position. With respect to the pending rejections, the term "anti-CD3 active compound" is used throughout the specification and prosecution history to denote non-mitogenic, anti-CD3 antibodies or fragments thereof. Thus, one of skill in the art would have recognized that appellant's possessed the claimed invention at the time of filing. In terms of practicing the claimed invention, the nonobese diabetic (NOD) mouse is accepted by practitioners as reasonably correlating to insulin-dependent diabetes mellitus (IDDM) in humans. Accordingly, the sufficiency of the model in terms of predicting

human efficacy cannot be rejected out of hand. Furthermore, there is no objective evidence of record that one skilled in the art would have to undertake anything more than the kind and amount of experimentation that is endemic to this field. Regarding the inventiveness of the claimed methods, obviousness cannot be predicated on what is unknown. Therefore, Chatenoud *et al.* (1994) cannot render obvious the claimed methods because there is no objective evidence of record that an artisan at the time of the invention recognized in Chatenoud's disclosure even the possibility of treating animals with overt automimmune disease with $F(ab')_2$ fragments of anti-CD3 antibody. Chatenoud's administration of $F(ab')_2$ fragments of an anti-CD3 antibody to non-diabetic mice treated with cyclophosphamide foretold nothing about the medicament's effectiveness or durability in treating humans, or any other mammal, with established auto-immune disease.

IX. ARGUMENT

A. USPTO rules compel a reversal of the examiner's position

Appellants have actively prosecuted the instant case for over five years. After Examiner VanderVegt, who originally handled the case, refused to recognize the erroneous nature of his rejections, applicants appealed their case to the Board of Patent Appeals and Interferences. The Board reversed an obviousness rejection of all appealed claims, based on the combination of Racadot *et al.*, Gussow *et al.*, and Chatenoud *et al.* Under 37 C.F.R. §1.196(b), the Board also enunciated a new lack-of-novelty rejection of claim 1, based on Chatenoud *et al.* Thus, the Board returned only an anticipation rejection of claim 1 to Examiner Saunders, who had taken over the case by that time.[1]

While disagreeing with the Board's inherency theory of anticipation and its characterization that appellants had "grouped the claims as standing or falling together," appellants opted to advance prosecution by qualifying claim 1 to recite methods of treating "humans," rather than "mammals." As Examiner Saunders acknowledged, this amendment

[1] The Board decided that claims 2, 4-7, 9-13 and 16-18 "fall together with claim 1" because appellants had "grouped the claims as standing or falling together." Decision at page 7, lines 1-3. Because the pending rejection was raised for the first time by the Board, however, appellants could not have grouped the claims in light of the new grounds for rejection. A request for clarification was filed at the Board on March 7, 2002.

overcame the Board's only rejection.[2] Thus, the prosecution of the instant application should have reached a denouement.

In violation of 37 C.F.R. §§ 1.196 and 1.198, however, the examiner reopened prosecution. Without authority, the examiner issued a new written description rejection under 35 U.S.C. §112, ¶1. In particular, the examiner now questions the use of the term "anti-CD3 active compounds," which was specifically approved by the examiner's predecessor and previously acknowledged by Examiner Saunders himself.[3] In addition, the examiner issued a new enablement rejection under 35 U.S.C. §112, ¶1. Finally, the examiner levied two obviousness rejections based on an alleged *inherent* disclosure in Chatenoud *et al.*, thereby managing to simultaneously violate the law both procedurally and substantively.

Simply put, the examiner lacked the authority to issue the pending rejections.[4] According to the MPEP "a new ground of rejection raised by the Board [under 37 C.F.R. 1.96(b)] does not reopen the prosecution except as to that subject matter to which the new rejection was applied."[5]

In an effort to force the PTO to comply with its rules, appellants filed a Petition to Invoke the Supervisory Authority of the Commissioner. The examining core denied the petition. In justifying its decision, the core asserted that 1) introduction of claim amendments following a new ground of rejection raised by the Board under 37 C.F.R. 1.96(b) enables an examiner to reconsider all issues pertaining to patentability and 2) a Group Director can reopen prosecution of any case decided by the Board.[6] The core cited no authority for its first justification, and appellants are aware of no such decree. Regarding the second justification, 37 C.F.R. §1.198 and MPEP § 1002.02(c)[7] grants a group director the authority to reopen prosecution after a decision by the Board. A director cannot act arbitrarily or capriciously, however. Indeed, the rule mandates that prosecution

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[2] Office Action dated June 11, 2002, page 3, ¶3.

[3] As Examiner VanderVegt's supervisor, Examiner Saunders signed off on the earlier actions.

[4] *See* 37 C.F.R. §§ 1.196 and 1.198.

[5] MPEP §1214.01 (Aug. 2001), pg. 1200-34.

[6] Petition Decision, dated January 24, 2003, pg. 2, 4th ¶.

[7] MPEP § 1002.02(c) (Aug. 2001) pg. 1000-7.

be reopened "only for the consideration of matters not already adjudicated, sufficient cause being shown."[8]

Appellants assert that insufficient cause has been shown for reopening prosecution of the instant case. In fact, when reopening prosecution, the examining division presented no basis or justification whatsoever. Such unrestrained actions are the hallmark of capricious decision-making in a bureaucracy.

Furthermore, the introduction of new rejections at this stage epitomizes piecemeal examination, which, according to the Patent Office's examination guidelines, is prohibited.[9] That is, all "[m]ajor technical rejections . . . should be applied where appropriate even though there may be a seemingly sufficient rejection on the basis of prior art."[10] In promulgating the rule, the Patent Office recognized the necessity to streamline the examination process so as to limit prosecution costs and to preserve patent term.

Yet the examiner in the instant case has issued new enablement and written description rejections after five years of prosecution and an appeal to the Board. This is exactly the type of conduct barred by the guidelines.

In summary, the pending rejections should be overruled because they were issued without authority and in contradiction to the Patent Office's own rules.

B. One of skill in the art would have recognized that appellants possessed the claimed invention at the time of filing

The claimed invention relates to treating spontaneous and ongoing autoimmune disease by administering a therapeutically effective amount of a non-mitogenic, anti-CD3-active compound to a human and thereby achieving a permanent disease remission, through the induction of antigen-specific tolerance.

The examiner asserts that "[a]pplicant[s] did not possess the genus of "anti-CD3 active compounds."[11] This assertion is erroneous as a matter of law, however.

Throughout the specification[12] and prosecution history,[13] the term "anti-CD3 active compound" is used to denote non-mitogenic, anti-CD3 antibodies or fragments thereof.

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[8] 37 C.F.R. §1.198

[9] *See* MPEP §707(g) (Aug. 2001), pg. 700-100.

[10] *Id.*

[11] Office Action mailed March 18, 2003, pg. 2, 2nd ¶.

[12] *See e.g.* Specification, pg. 3, ln. 11-25; pg. 5, ln. 1-5, describing anti-CD3 active principles.

[13] *See e.g.* Appeal Brief filed November 15, 1999.

These compounds, well-known at the time of the invention, share similar structural and functional characteristics. Structurally, all anti-CD3 active compounds take the form of an immunoglobulin or fragment thereof, regardless of whether they are covalently linked with a non-immunoglobulin protein. Functionally, all anti-CD3 active compounds bind an antigen of the CD3 receptor complex expressed upon T cells.

Thus, one of skill in the art, in view of the specification and prosecution history, would have recognized that appellants possessed the claimed invention at the time of filing. Accordingly, the examiner's rejection should be overruled.

C. An artisan could practice the invention without undue experimentation in view of the specification's disclosure

According to the examiner, "[a]pplicant has not enabled the treatment of established autoimmune disease in humans."[14] So stating, the examiner has interpreted the patent statute as requiring proof of human efficacy for claims concerning methods of treatment. This interpretation is erroneous as a matter of law, however.

It is well-established that "Title 35 does not demand that such human testing occur within the confines of Patent and Trademark Office (PTO) proceedings."[15] Like the Board in *In re Brana*, the examiner "confuses the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption."[16]

Under §112, the application must explain how to "make and use" the claimed invention. The courts have interpreted this statute to mean that the specification must teach the skilled artisan how to practice the invention without undue experimentation.[17] Thus, the test is not whether experimentation is necessary but whether any experimentation would be undue in view of what is typical in the area.[18]

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[14] Office Action mailed March 18, 2003, pg. 2, 3rd ¶.

[15] *Scott v. Finney*, 34 F.3d 1058, 1063, 32 USPQ2d 1115, 1120 (Fed. Cir. 1994).

[16] *In re Brana*, 51 F.3d 1560, 1567, 34 USPQ2d 1436, 1442 (Fed. Cir. 1995).

[17] *See Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986).

[18] *See* MPEP §2164.01 (Feb. 2003) at pages 2100-178 to 2100-179.

In levying an enablement rejection, the examiner has the burden of establishing why he believes that the scope of the claims is not adequately enabled by the specification.[19] Appellants respectfully submit that the examiner has failed to meet this burden.

The examiner has proffered no evidence whatsoever that one skilled in the art would have needed an undue amount or type of experimentation to make and use the claimed invention. Rather, he merely states that "the office does not consider it clear that the diabetes in NOD mice has the same mechanistic basis as human diabetes."[20]

Devoid as it is of any factual basis, this statement cannot satisfy the office's burden in this context. Indeed, when an animal model is recognized as correlating to a specific condition, the office must accept the correlation unless the examiner marshals evidence to the contrary.[21] "Even with such evidence, the examiner must weigh the evidence for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition."[22] Also, a "rigorous or an invariable exact correlation is not required."[23]

Appellants utilized nonobese diabetic (NOD) mice to evaluate their invention. They selected the NOD mouse precisely because it had been studied extensively as a model of insulin-dependent diabetes mellitus (IDDM).[24] The model is a useful tool in the identification of candidate therapies due to the distinct similarities between human and murine autoimmune diabetes progression.[25] Similarities include, for example, (i) the strong genetic association of autoimmune diabetes in both species with their respective loci of the major histocompatibility complex, (ii) the presence of insulitis (lymphocytic infiltration) in the pancreatic islets of both species, and (iii) the development of a multi-specificity B cell response and secretion of auto-antibodies against islet cell antigens. Both diabetic humans and NOD mice undergo a similar disease process with distinct similarities in the disease

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[19] *See* MPEP §2164.04 (Feb. 2003), pg. 2100-183

[20] Office Action mailed March 18, 2003, pg. 3, 1st ¶.

[21] *See* MPEP §2164.02(g) (Feb. 2003), pg. 2100-81.

[22] *Id.* (citing *In re Brana*, 51 F.3d 1560, 34 USPQ2d 1436 (Fed. Cir. 1995)(reversing the PTO decision based on finding that in vitro data did not support in vivo applications).

[23] *Id.*

[24] Castano *et al.*, *Annu. Rev. Immunol.*, 8:647 (1990); Bach, *Endocr. Rev.*, 15:516 (1994).

[25] Castano *et al.*, (1990); Prochazka *et al.*, *Science*, 237(4812):286-9 (1987).

pathology.[26] Furthermore, the disease progression is mediated by similar types of immune cells where identical self-proteins (autoantigens) are the target of B and T cell activities in both species. Moreover, therapeutic regimens validated in the NOD mouse have shown efficacy in human clinical trials for the therapeutic treatment of autoimmune disease.[27]

As one skilled in the art would accept the NOD mouse as reasonably correlating to IDDM, the examiner erred as a matter of law in dismissing out of hand the status of that model as a "working example." The examiner's rationale is legally erroneous, too, because there is no objective evidence of record that one skilled in the art, at the time of the invention, would have to undertake anything more than the kind and amount of experimentation that is endemic to this field. Accordingly, the rejection should be overruled.[28]

D. Unrecognized benefits inherent in a prophylactic protocol cannot support an obviousness rejection for a new method of treatment

In its previous decision, the board concluded that the method used by Chatenoud *et al.* (1994) **inherently** yielded the same result as the previously claimed invention. *See* Decision on Appeal, pg. 6, ¶2. While disagreeing with the Board's conclusion,[29] appellants amended claim 1 to recite "human" rather than "mammal," thereby removing Chatenoud *et al.* as an anticipatory reference.

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[26] Bowman *et al.*, *Immunol. Today*, 15(3):115-20 (1994), in particular page 115, 1st ¶

[27] Elias *et al. Diabetes*, 46(5):758-64(1997); Bockova *et al.*, *J. Autoimmun.*, 10(4):323-9 (1997); Elias *et al.*, *Diabetologia*, 43:[Suppl 1]: 410 [p.A105] (2000).

[28] In addition, Appellants note that a recent publication demonstrates that the inventive treatment, *i.e.* short-term, low dose administration of non-mitogenic anti-CD3 active compounds, induces in diabetic humans a durable restoration of self-tolerance. *See* Herold *et al.*, *N. Engl. J. Med.* 346(22):1692-98 (2002). The examiner has refused to admit the article after Final Rejection.

[29] Indeed, the Federal Circuit's predecessor reversed a Board finding of ***anticipation*** based on inherency, when, as here, the determinative feature was not detectable in the prior art. *In re Seaborg*, 328 F.2d 996, 999, 140 USPQ 662, 665 (CCPA 1964) (adopting appellant's summary: "The possibility that although a minute amount of americium may have been produced in the Fermi reactor, it was not identified (nor could it have been identified) would preclude the application of the Fermi patent as a reference to anticipate the present invention."). As noted in the first Appeal Brief and at the related Oral Proceeding, Chatenoud's experiment that employed an accelerated model of insulin-dependant diabetes mellitus (IDDM) was incapable of demonstrating the long-term effectiveness of administration of non-mitogenic anti-CD3 active compounds. The cyclophosphamide (CY) used to accelerate the onset of IDDM in mice also accelerates their death, such that treated mice typically die within 50 days of CY administration. As in *Seaborg*, therefore, the determinative features of appellants' presently claimed invention were undetectable in the cited art.

The examiner asserts that "[m]erely changing the claims from "mammal" to "human" treatment methods does not overcome what was found by the [Board]."[30] This assertion is erroneous as a matter of law, however.

Chatenoud administered $F(ab')_2$ fragments of an anti-CD3 antibody to **non-diabetic** mice to determine the treatment's efficacy in **preventing** IDDM. Chatenoud taught that short-term, low-dose administration of $F(ab')_2$ fragments of an anti-CD3 antibody to non-diabetic mice successfully prevented the onset of diabetes.[31] While the authors acknowledged that an unidentified sub-population (alleged to be approximately 15-20%) of the treated mice were already diabetic upon administration of therapy, no efforts were made to identify the subgroup's members. Accordingly, Chatenoud provides no information on the effect of the anti-CD3 $F(ab')_2$ fragments on diabetic mice. Instead, the article taught only that therapy comprising $F(ab')_2$ fragments of an anti-CD3 antibody effectively **prevents** the onset of diabetes in **non-diabetic** mice.[32]

By contrast, the claimed invention is directed to a method of employing $F(ab')_2$ fragments of an anti-CD3 antibody to **treat** humans with **established auto-immune disease** to achieve a permanent disease remission, *i.e.*, a durable restoration of self-tolerance. Nothing in Chatenoud suggested such a treatment. Moreover, contrary to the examiner's assertion, the mere fact that an unidentified subgroup of Chatenoud's test animals were diabetic cannot render, as a matter of fact, the claims obvious based on the inherency doctrine. As has been previously demonstrated within the art using NOD mice, the preventative effect of an immunomodulatory agent in autoimmune diabetes is maximized during the earliest stages of disease progression (*i.e.*, at the earliest age interval of an NOD mouse), but is nullified by disease onset. In particular, this has been illustrated with the administration of anti-CD28 antibodies, where neonatal administration completely abrogated diabetes onset while post-onset therapy with the same agent failed to ameliorate the disease.[33] Similarly, oral administration of Linomide (quinoline-3-carboxamide) completely prevents diabetes onset among NOD mice when treated at a very young age

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[30] Office Action mailed March 18, 2003, pg. 3, last ¶.

[31] *See* Figures 2C and 2D of Chatenoud *et al.*, *Proc. Natl. Acad. Sci. USA*, 91:123-127 (1994).

[32] *Id.*

[33] Arreaza *et al.*, *J. Clin. Invest.*, 100(9):2243-53 (1997).

prior to disease onset, while administration that is delayed to advancing disease stages shows little efficacy.[34]

Moreover, as a matter of law, "[i]nherency and obviousness are distinct concepts."[35] To support an obviousness rejection, an inherent teaching must have been obvious to those skilled in the art at the time of the invention.[36] According to the Federal Circuit,

> The mere fact that a certain thing *may* result from a given set of circumstances is not sufficient to establish inherency. That which may be inherent is not necessarily known. Obviousness cannot be predicated on what is unknown. Such a retrospective view of inherency is not a substitute for some teaching or suggestion supporting an obviousness rejection.[37]

In the instant case, there is no objective evidence of record that an artisan at the time of the invention recognized in Chatenoud's disclosure even the possibility of treating mammals with overt autoimmune disease with $F(ab')_2$ fragments of an anti-CD3 antibody could, let alone understood the physiological consequences of the treatment with the certainty required for the examiner to wield the inherency doctrine. All of Chatenoud's data and commentary regarding "reversing overt disease" dealt exclusively with treatment comprising anti-CD3 antibodies. Chatenoud neither identified the alleged diabetic subjects receiving anti-CD3 antibody fragments nor presented any indication that the prophylactic treatment effected self-tolerance. Furthermore, as noted above, Chatenoud's experiment was incapable of demonstrating the long-term effectiveness of administration of non-mitogenic anti-CD3 active compounds because the cyclophosphamide (CY) used to accelerate the onset of IDDM in mice also accelerates their death, such that treated mice typically die within 50 days of CY administration.[38] Thus, as a matter of fact, the efficacy of Chatenoud's use of $F(ab')_2$ fragments of an anti-CD3 antibody in effecting a durable restoration of self-tolerance was unknowable. Moreover, the only evidence in the record on this point suggests "that a person knowledgeable in immunology, circa 1997, would not

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[34] Gross *et al.*, *Diabetologia*, 37:1195–1201 (1994).

[35] *Kloster Speedsteel AB v. Crucible Inc.*, 793 F.2d 1565, 1576 (Fed. Cir. 1986).

[36] *Id.*

[37] *In re Rijckaert*, 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993) (overruling the Board's inherency-based obviousness rejection)(citations omitted).

[38] *See* footnote 29.

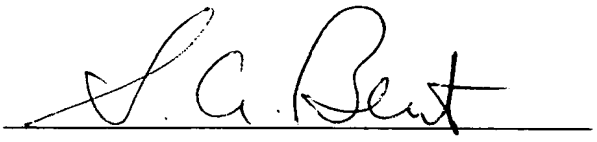
have expected a durable, antigen-specific unresposiveness to result from administering anti-CD3 antibody fragments."[39]

As a matter of law, therefore, the examiner has erred in asserting that Chatenoud's disclosure inherently renders the appealed claims obvious. Accordingly, the rejection should be overruled.

Respectfully submitted,

Date

18 June 2007



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[39] Declaration of Dr. Terry Strom, submitted with first Appeal Brief, filed November 15, 1999.

Appendix A

WE CLAIM

1. A method of treating spontaneous and ongoing auto-immune diseases in humans, comprising administering to a human, in need of such a treatment, a therapeutically effective amount of one or more non mitogenic anti-CD3 active compounds to achieve permanent disease remission through the induction of antigen-specific unresponsiveness, i.e. immune tolerance.

2. The method of claim 1, wherein said non mitogenic anti-CD3 active compound is a non mitogenic anti-CD3 antibody.

4. The method of claim 1, wherein said non mitogenic anti-CD3 active compound is a non mitogenic anti-CD3 monoclonal antibody.

5. The method of claim 1, wherein said non mitogenic anti-CD3 active compound is a non mitogenic anti-CD3 monoclonal antibody $F(ab')_2$ fragment.

6. The method of claim 1, wherein said non mitogenic anti-CD3 active compound is highly purified, endotoxin-free.

7. The method of claim 4, wherein said monoclonal antibody is selected from the group consisting of murine or humanized antibody.

9. The method of claim 1, wherein said auto-immune disease is diabetes.

10. The method of claim 1, wherein said auto-immune disease is rheumatoid arthritis.

11. The method of claim 1, wherein said auto-immune disease is multiple psoriasis.

12. The method of claim 1, wherein said auto-immune disease is multiple sclerosis.

13. The method of claim 1, wherein said active compound is administered by injectable route.

16. The method of claim 1, wherein said non mitogenic anti-CD3 compound is a $F(ab')_2$ fragment.

17. The method of claim 13, wherein said active compound is in the form of an injectable solution delivering between 5 and 20 mg of active compound per day.

18. The method of claim 17, wherein said active compound is in the form of an injectable solution delivering between 5 and 10 mg of active compound per day.

Appendix B

Arreaza *et al.*, "Neonatal Activation of CD28 Signaling Overcomes T Cell Anergy and Prevents Autoimmune Diabetes by an IL-4-Dependent Mechanism," *J. Clin. Invest.*, 100: 2243-2253 (1997).

- Bach, "Insulin-Dependent Diabetes Mellitus as an Autoimmune Disease," *Endocrine Reviews*, 15: 516-542 (1994).

- Bockova *et al.*, "Treatment of NOD Diabetes with a Novel Peptide of the hsp60 Molecule Induces Th2-type Antibodies," *J. Autoimmunity*, 10: 323-329 (1997).

Bowman *et al.*, "Treatment of Diabetes in the NOD Mouse: Implications for Therapeutic Intervention in Human Disease," *Immunology Today*, 15: 115-120 (1994).

- Castano *et al.*, "Type-I Diabetes: A Chronic Autoimmune Disease of Human, Mouse, and Rat," *Ann. Rev. Immunol.*, 8: 647-679 (1990).

Elias *et al.*, "Hsp60 Peptide Therapy of NOD Mouse Diabetes Induces a Th2 Cytokine Burst and Downregulates Autoimmunity to Various β-Cell Antigens," *Diabetes*, 46: 758-764 (1997).

- Elias *et al.*, "Animal Models for Type 1 and Type 2 Diabetes: Preservation of C-Peptide and Induction of TH2 Cytokines in Type 1 Diabetes Newly Diagnosed Adults Vaccinated with DIAPEP277," *Diabetologia*, 43: 410 (2000)

Gross *et al.*, "Prevention of Diabetes Mellitus in Non-Obese Diabetic Mice by Linomide, a Novel Immunomodulating Drug," *Diabetologia*, 37: 1195-1201 (1994).

- Herold *et al.*, "Anti-CD3 Monoclonal Antibody in New-Onset Type 1 Diabetes Mellitus," *N. Engl. J. Med.*, 346: 1692-1698 (2002).

- Prochazka *et al.*, "Three Recessive Loci Required for Insulin-Dependent Diabetes in Nonobese Diabetic Mice," *Science*, 23: 286-289 (1987).

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Neonatal Activation f CD28 Signaling Ov rcomes T Cell An rgy and Pr vents Autoimmune Diabetes by an IL-4–d pendent Mechanism

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Abstract

Optimal T cell responsiveness requires signaling through the T cell receptor (TCR) and CD28 costimulatory receptors. Previously, we showed that T cells from autoimmune nonobese diabetic (NOD) mice display proliferative hyporesponsiveness to TCR stimulation, which may be causal to the development of insulin-dependent diabetes mellitus (IDDM). Here, we demonstrate that anti-CD28 mAb stimulation restores complete NOD T cell proliferative responsiveness by augmentation of IL-4 production. Whereas neonatal treatment of NOD mice with anti-CD28 beginning at 2 wk of age inhibits destructive insulitis and protects against IDDM by enhancement of IL-4 production by islet-infiltrating T cells, administration of anti-CD28 beginning at 5–6 wk of age does not prevent IDDM. Simultaneous anti–IL-4 treatment abrogates the preventative effect of anti-CD28 treatment. Thus, neonatal CD28 costimulation during 2–4 wk of age is required to prevent IDDM, and is mediated by the generation of a Th2 cell–enriched nondestructive environment in the pancreatic islets of treated NOD mice. Our data support the hypothesis that a CD28 signal is requisite for activation of IL-4–producing cells and protection from IDDM. (*J. Clin. Invest.* 1997. 100:2243–2253.) Key words: diabetes mellitus • insulin dependent • mice, inbred NOD • islets of Langerhans • Th1 cells • Th2 cells

Introduction

Insulin-dependent diabetes mellitus (IDDM)[1] is a polygenic multifactorial autoimmune disease heralded by T cell infiltration of the pancreatic islets of Langerhans (insulitis) and progressive T cell–mediated destruction of insulin-producing β cells (1–3). CD4+ T helper cells are required for the adoptive transfer of IDDM into recipient neonatal NOD mice or immunodeficient NOD.Scid mice (4–6). Cooperation between CD4+ and CD8+ T cells is required to initiate IDDM, and islet β cell destruction is CD4+ T cell–dependent (7, 8). Current evidence suggests that the CD4+ effector cells of IDDM in NOD mice are Th1 cells that secrete IL-2, IFN-γ, and TNF-α, and that the regulatory CD4+ cells are Th2 cells that secrete IL-4, IL-5, IL-6, IL-10, and IL-13 (9–11).

We previously discovered that, beginning at 3–5 wk of age, T cell receptor (TCR) ligation induces proliferative hyporesponsiveness of NOD thymic and peripheral T cells, which is mediated by reduced IL-2 and IL-4 production (12–14). Decreased IL-4 production by human T cells from patients with new-onset IDDM has also been demonstrated recently (15). Whereas addition of IL-4, a Th2-type cytokine, potentiates IL-2 production and completely restores NOD T cell proliferative responsiveness, addition of IL-2, a Th1-type cytokine, even at high concentrations, only partially restores NOD T cell responsiveness. These findings suggest that Th2 cells may be compromised in function to a greater extent than Th1 cells in NOD mice, and raise the possibility that Th2 cells require a higher threshold of activation than Th1 cells in these mice. IL-4 not only restores NOD T cell responsiveness in vitro, but prevents insulitis and IDDM when administered in vivo to prediabetic NOD mice (13) and when transgenically expressed in pancreatic β cells (16). The proliferative hyporesponsiveness of regulatory Th2 cells in NOD mice may favor a Th1 cell–mediated environment in the pancreas of these mice, and lead to a loss of immunological tolerance to islet β cell autoantigens. This possibility is consistent with the notion that restoration of the balance between Th1 and Th2 cell function may prevent IDDM (9, 10, 17).

Optimal T cell activation requires signaling through the TCR and CD28 costimulatory receptor (18–20). Cross-linking of the TCR/CD3 complex in the absence of a CD28-mediated costimulatory signal induces a proliferative unresponsiveness that is mediated by the inability of T cells to produce IL-2 (21). CD28 costimulation prevents proliferative unresponsiveness in Th1 cells by augmenting the production of IL-2, which in turn promotes IL-4 secretion by T cells (22). The costimulatory pathway of T cell activation involves interaction of CD28 with its ligands B7-1 and B7-2 on an antigen-presenting cell (APC), with B7-2 considered as the primary ligand for CD28 (23–26). When costimulation is blocked by either CTLA4-Ig or by anti-B7-1 or anti B7-2 mAbs, differential effects on the incidence of various autoimmune diseases (e.g., IDDM) and on the development of Th1 and Th2 cells are observed (27, 28). Furthermore, in vivo studies have demonstrated that generation of Th2 cells is more dependent upon the CD28-B7 pathway than is the

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1. *Abbreviations used in this paper:* APC, antigen-presenting cell; BGL, blood glucose level; GAD, glutamic acid decarboxylase; IDDM, insulin-dependent diabetes mellitus; TCR, T cell receptor.

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priming of Th1 cells, and suggest that the development of Th subsets in vivo may be influenced by limiting CD28-B7 costimulation (29, 30). These conclusions were recently confirmed using the CTLA-4Ig transgenic and CD28-deficient mouse models (31–33). Analyses of human Th2 cell development have yielded results similar to those observed in the mouse (34–36). Interestingly, interactions between CD28 and its B7-2 ligand are essential for costimulation of an IL-4–dependent $CD4^+$ T cell response, and IL-4 increases B7-1 and B7-2 surface expression on certain professional APCs (e.g., Langerhans cells) and B cells (26, 37, 38). Thus, NOD APCs may optimally activate islet β cell autoreactive $CD4^+$ effector T cells, but not regulatory $CD4^+$ T cells (39, 40). Deficient CD28 costimulation may lower the ability of NOD APCs to stimulate regulatory Th2 cells without compromising their ability to stimulate autoreactive effector Th1 cells.

In this study, we show that anti-CD28 mAb–mediated costimulation completely restores the proliferative responsiveness of NOD thymocytes and peripheral T cells by augmenting their levels of secretion of IL-2 and IL-4. This result raised the possibility that treating NOD mice with a CD28 agonist, which can stimulate marked IL-4 secretion and is more stable than IL-4 in vivo, may be more efficacious than IL-4 in protecting NOD mice from IDDM. NOD mice were therefore treated with an anti-CD28 mAb to determine whether CD28 costimulation protects them from IDDM. We demonstrate that anti-CD28 treatment effectively prevents destructive insulitis and protects from IDDM in NOD mice, provided that treatment is performed at a sufficiently early age (2–4 wk) during neonatal life. Our data support the hypothesis that NOD APCs may not provide a sufficient CD28 costimulation signal during the inductive phase of development of IDDM, and that this CD28 signal is requisite for activation of IL-4–producing NOD Th2 cells and protection from IDDM.

Methods

Mice. Our NOD/Del mouse colony was bred and maintained in a specific pathogen-free facility at The John P. Robarts Research Institute (London, ON). Diabetes incidence among females in our NOD colony is presently 40–50% at 15 wk of age and 80–90% by 25 wk. NOD.Scid mice generously provided by Dr. L. Shultz (The Jackson Laboratory, Bar Harbor, ME) were bred in our colony and used as recipients in T cell transfer experiments. The age- and sex-matched BALB/c mice used as controls in the in vitro T cell proliferation experiments were also bred in our colony.

Anti-CD28 mAb treatment. In the first experiment, either anti-CD28 mAb (50 μg), purified by protein G affinity chromatography (Pharmacia Biotech, Uppsala, Sweden) of supernatants from 37.51 hybridoma cells secreting hamster antimurine CD28 mAbs (41) (kindly provided by Dr. J. Allison, University of California, Berkeley, CA), or control hamster IgG (50 μg, Bio/Can Scientific, Mississauga, ON) was administered intraperitoneally every other day to female NOD mice (n = 20/group, randomized from 10 different litters) from 2 to 4 wk of age. These mice were then boosted at 6, 7, and 8 wk of age. In a second experiment, female NOD mice (n = 10–12/group randomized from 3 to 4 different litters) received anti-CD28 mAb (50 μg) plus either anti–IL-4 mAb (42) (50 μg, 11B11) or control rat IgG (50 μg; Bio/Can Scientific, Mississauga, ON), or anti–IL-4 mAb (50 μg, 11B11) alone, according to the same schedule used in the first experiment. In a third experiment NOD mice (n = 10/group, randomized from five different litters) received the same treatment starting at 5 wk of age. Blood glucose levels (BGL) were measured weekly with a Glucometer Encore (Miles/Bayer, Toronto, ON). Animals with BGL > 11.1 mmol/liter (200 mg/dl) during two consecutive wk were considered diabetic.

Histopathology analysis. Mice were harvested periodically during the course of anti-CD28 or control treatment, and pancreatic tissue was removed, fixed with 10% buffered formalin, embedded in paraffin, and sectioned at 5-μm intervals. The incidence and severity of insulitis was examined by hematoxylin and eosin staining as well as insulin immunostaining. A minimum of 20 islets from each mouse were observed, and the degree of mononuclear cell infiltration was scored by two independent blinded observers using the following ranking: 0, normal; 1, periinsulitis (mononuclear cells surrounding islets and ducts, but no infiltration of the islet architecture); 2, moderate insulitis (mononuclear cells infiltrating < 50% of the islet architecture); and 3, severe insulitis (> 50% of the islet tissue infiltrated by lymphocytes and/or loss of islet architecture). Immunohistochemical detection of insulin was performed using a porcine anti–insulin antibody and avidin–biotin peroxidase technique (Dako Corp., Carpinteria, CA).

Cell proliferation and cytokine secretion. Splenocytes and thymocytes were isolated as described (13). Splenic T cells were isolated on T cell columns (R&D Systems, Minneapolis, MN) to a purity of ≥ 98%, as assayed by FACS analysis of CD3 cell surface expression. Cells (10^6/ml) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 10 mM Hepes buffer, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.05 mM 2-ME (all purchased from Gibco Laboratories, Grand Island, NY) with plate-bound 145-2C11 anti-CD3ε mAb (1/500 dilution of ascites; hybridoma kindly supplied by Dr. J. Bluestone, University of Chicago, Chicago, IL) in the presence or absence of various concentrations of the 37.51 anti-CD28 mAb. Cells were harvested after either 48 (splenocytes and T cells) or 72 h (thymocytes), and were then assayed for incorporation of [^{3}H]thymidine (1 μCi/well; Amersham, Oakville, ON) added during the last 18 h of culture. Islet-infiltrating cells were purified after isolation of pancreatic islets by collagenase P (Boehringer Mannheim, Laval, QC) digestion and centrifugation of the islets on a discontinuous Ficoll gradient. Free islets were hand-picked under a dissecting microscope to a purity of ≥ 95%, and purified islets were cultured for 24 h to allow emigration of lymphocytes from the islets. After culture harvest and isolation of viable lymphocytes by density gradient centrifugation on Lympholyte-M (Cedarlane Laboratories, Ltd., Hornby, ON), the cells were cultured for 48 h with anti-CD3 as above.

Culture supernatants were assayed for their concentration of cytokines by ELISA. IL-4 levels were interpolated from a standard curve using recombinant mouse IL-4 captured by the BVD4-1D11 mAb and detected by the biotinylated BVD6-24G2 mAb, while IFN-γ concentrations were measured using rmIFN γ, the R4-6A2 mAb, and biotinylated XMG1.2 mAb (all obtained from PharMingen, Mississauga, ON). Standard curves were linear in the range of 20–2,000 pg/ml. In some experiments, the relative levels of IL-2 and IL-4 secreted were quantified in a bioassay using the IL-2 dependent CTLL-2 T cell line (43) and IL-4 dependent CT.4S T cell line (44) (supplied by Dr. W.E. Paul, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, Bethesda, MD). Twofold serial dilutions of test supernatants were added to CTLL-2 cells (1.5×10^4) and CT.4S cells (5×10^3), which were cultured for 24 and 48 h, respectively, in flat-bottomed 96-well plates. Cell proliferation was assessed by addition of [^{3}H]thymidine for 8 h before termination of culture, and [^{3}H]thymidine incorporation was determined as above. To assess the ability of either anti–IL-2 (S4B6) (45) or anti–IL-4 (11B11) mAbs to inhibit CD28 costimulation of anti-CD3 activated T cells, the percentage inhibition of CD28 costimulation was calculated as follows:

$$\text{Percentage of inhibition} = 1 - \frac{([S\text{-}U] \text{ in the presence of an inhibitor mAb})}{([S\text{-}U] \text{ in the absence of an inhibitor mAb})} \times 100$$

where S is the amount of CD28 costimulation-induced proliferation (or cytokine production) of anti-CD3 plus anti-CD28–stimulated

cells, and U is the amount of proliferation (or cytokine production) of cells activated by anti-CD3 alone.

Intrapancreatic cytokine analysis. Intrapancreatic IL-4 and IFN-γ concentrations in tissue samples were quantified as described (46). In brief, pancreata were isolated and snap frozen in liquid nitrogen. Upon analysis, the samples were homogenized and sonicated in protease inhibitor–buffered cocktail followed by filtration through 1.2-μm filters (Gelman Sciences, Inc., Ann Arbor, MI). The filtrates were analyzed for IL-4 and IFN-γ concentrations by ELISA, and the ELISA results were normalized relative to the total amount of protein per pancreas and recorded as ng/mg tissue.

Glutamic acid decarboxylase (GAD) antibody ELISA. The presence of anti-GAD antibodies in collected sera was determined by ELISA as previously described (47). In brief, sera samples were added at appropriate dilutions to plates coated with murine GAD_{67} (10 μg/ml). Using alkaline phosphatase–conjugated goat anti–mouse isotype (IgG1 or IgG2a) antibodies with *p*-nitrophenylphosphate disodium in diethylamine buffer (substrate) the optical density was read at 405 nm to determine the relative amount of the individual anti-GAD isotype. All sera were titrated at 1:20, 1:40, 1:80, and 1:160 dilutions for anti-GAD_{67} antibodies. Since we found significant differences between the IgG1 and IgG2a ratio at the 1:20 dilution between treated and untreated mice, all sera were tested for the specific isotypes (IgG1 and IgG2a) at the 1:20 dilutions.

Adoptive cell transfer. Female NOD.Scid mice (n = 5/group) 6–8 wk of age were each injected intraperitoneally with splenic T cells (10^7) from prediabetic female NOD mice previously treated with anti-CD28 mAb or control Ig. The recipients were followed for a maximum of 12 wk after transfer, and BGL were monitored weekly.

Flow cytometry analysis. Splenic T cells and thymocytes (10^5) were suspended in 0.1% BSA and PBS/0.001% NaN_3, and were then incubated for 30 min at 4°C with various FITC- or PE-conjugated mAbs against different murine lymphocyte subpopulations and functional markers, including CD3, CD4, CD8, CD19, CD25, CD69, CD44, L-selectin, CD40, LFA-1, B7-1, and B7-2 (PharMingen). Isotype-matched (Ig) antibodies were used as negative controls. Cell fluorescence was analyzed using a FACScan and Lysis II software (both from Becton-Dickinson, San Jose, CA).

Results and Discussion

CD28 costimulation restores NOD T cell proliferative responsiveness. A primary role of CD28 costimulation is the augmentation of IL-2 production by activated T cells. If exogenous IL-2 is present at a high concentration, specific intracellular signals derived from CD28-mediated costimulation are unnecessary for maximum Th1 proliferation and optimal Th2 responsiveness to IL-4 (22). Addition of IL-2 can also overcome the inability of Th1 cells to proliferate (21). Nonetheless, we previously showed that exogenous IL-2, even when added at high concentrations, only partially restores TCR-induced NOD thymocyte and peripheral T cell proliferative responsiveness due to its inability to restore a normal level of IL-4 production (13). In contrast, exogenous IL-4 completely restores the TCR-mediated responsiveness of NOD T cells, and this is associated with increased IL-2 production by these T cells. These data raise the possibility that the costimulatory signal transduced by CD28 on NOD T cells may be insufficient to stimulate optimum NOD T cell activation.

To test this possibility, we assayed the ability of an anti-CD28 mAb to augment the costimulation signal provided by NOD APCs to NOD T cells, and stimulate the TCR-mediated in vitro proliferation of NOD and BALB/c thymocytes. 8-wk-old mice were used, since the trait of T cell proliferative hyporesponsiveness is readily detectable in NOD mice at this age (12). Fig. 1 *A* shows that CD28 costimulation provided by anti-CD28 markedly enhances the anti-CD3–induced proliferative responses of NOD and BALB/c thymocytes, yielding 19.5- and 5.6-fold increases (at the highest concentration of anti-CD28) in these responses, respectively. Note also that the response to anti-CD3 mAb alone is significantly higher in control BALB/c than NOD thymocytes (Fig. 1, *A* and *E*), confirming the NOD T cell proliferative hyporesponsiveness reported in our previous work (12, 13). When quiescent NOD and BALB/c thymocytes were stimulated by anti-CD28 in the absence of anti-CD3 (or anti-TCR αβ), however, a low level of proliferation was observed which was equivalent to the basal proliferative response detected in the absence of any stimulus (data not shown). The negligible effect of anti-CD28 stimulation on T cell activation in the absence of TCR ligation agrees with the result predicted by a recently proposed model of T cell–APC interaction (18). Anti-CD28 mAb also significantly enhanced the NOD, and to a lesser extent the BALB/c, anti–CD3-induced splenic T cell proliferative response (Fig. 1 *B*). Thus, CD28 costimulation is able to restore NOD thymocyte and T cell proliferation to levels similar to the levels found in BALB/c and other control strains (Fig. 1, *A*, *B*, and *E*).

CD28 costimulation activates IL-2 and IL-4 production by NOD T cells. CD28 costimulation of IL-2 production enhances IL-4 production by T cells (22), and CD28 costimulation also induces the responsiveness of Th2 cells to IL-4 (48). We reasoned, therefore, that CD28 costimulation may restore NOD T cell responsiveness by augmenting production of not only IL-2, but also IL-4. Fig. 1 *C* demonstrates that anti-CD3 plus anti-CD28 costimulation significantly increases IL-2 production by both NOD (21.6-fold) and BALB/c (8.1-fold) thymocytes. In contrast, anti-CD28 significantly enhanced anti-CD3-stimulated IL-4 production by NOD (5.5-fold increase) but not BALB/c thymocytes (Fig. 1, *D* and *G*). This result may be due to higher anti-CD3 induced IL-4 production by BALB/c T cells without the requirement of costimulation. NOD mice may be deficient in generating differentiated Th2 cells, and therefore require CD28 costimulation for IL-4 production (49). A higher anti-CD3 stimulated production of IL-4 has also been found in other diabetes-resistant strains (our unpublished results [50]). The failure of TCR-stimulated CD4+ NOD thymocytes to produce IL-4 (13) likely facilitates detection of a significant increase in IL-4 production upon costimulation by anti-CD28. Note that CD28 costimulation augments the proliferative responsiveness as well as IL-2 and IL-4 production by NOD thymocytes to levels comparable to those of BALB/c thymocytes. This augmentation may occur by a CD28-mediated pathway that significantly enhances the differentiation and ability of NOD thymocytes to produce IL-4, which can subsequently stimulate T cell proliferation in an autocrine and/or paracrine fashion (51, 52). Our finding that IL-4 restores the proliferative responsiveness of NOD thymocytes by increasing their level of IL-2 production (13) agrees closely with the reported role for IL-4 in the stimulation of IL-2 production by mouse T cells in response to plate-bound anti-CD3 (53).

To examine whether CD28 costimulation of NOD thymocytes is dependent upon upregulation of IL-4 and/or IL-2 production, we assayed the capacity of anti-CD28 to costimulate anti–CD3-induced NOD and BALB/c T cell proliferative responses in the presence of anti-IL-4, anti-IL-2, or both of these mAbs. Anti-IL-4 inhibited ~ 33% of the CD28 costimulatory response of NOD thymocytes, but did not inhibit CD28

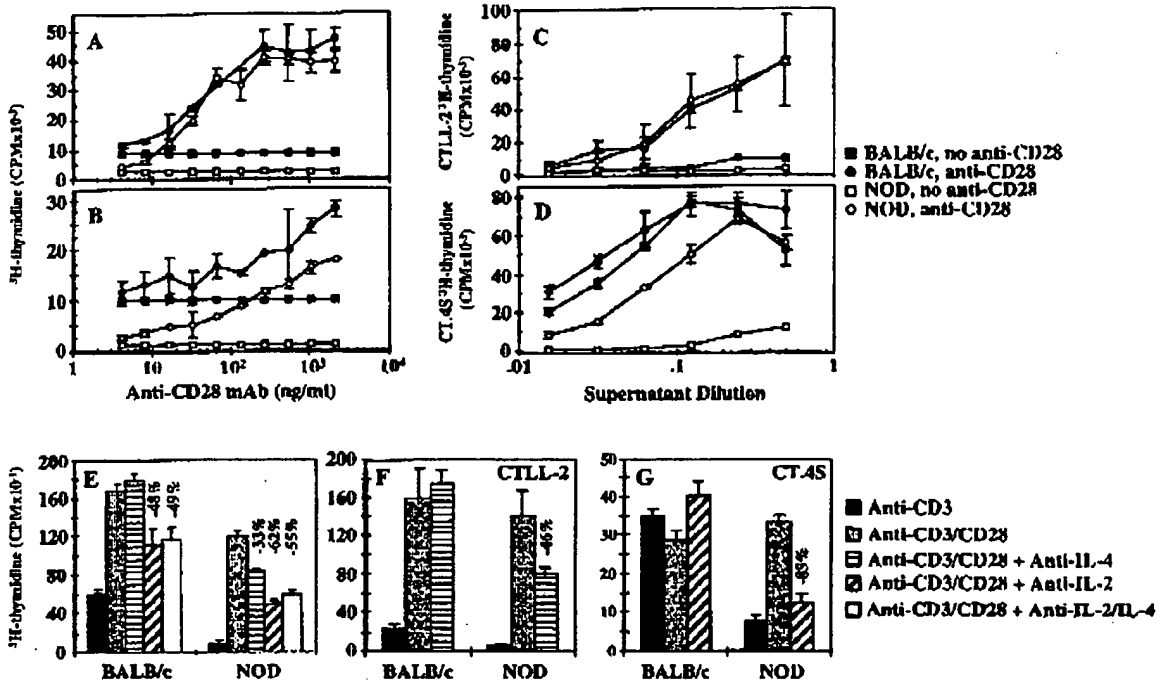

Figure 1. CD28 costimulation restores the proliferative responsiveness of activated NOD thymic and splenic T cells, and augments IL-2 and IL-4 production by activated NOD thymocytes. Thymocytes (*A*) and splenic T cells (*B*) from 8-wk-old NOD and control BALB/c mice were activated by plate-bound anti-CD3 in the absence (*squares*) or presence (*circles*) of varying dilutions (2 ng/ml–2 μg/ml) of soluble anti-CD28 mAb. Cell proliferation was determined by [^{3}H]thymidine incorporation. The results of triplicate cultures are expressed as the mean values±SD, and are representative of three different experiments. In *C* and *D*, NOD and BALB/c thymocytes from 8-wk-old mice were activated by plate-bound anti-CD3 in the absence (*squares*) or presence (*circles*) of 1 μg/ml soluble anti-CD28 mAb (optimal concentration). Culture supernatants were removed, diluted and assayed for their IL-2 (*C*) and IL-4 (*D*) content by stimulation of proliferation of the CTLL 2 and CT.4S T cell lines, respectively. The CTLL-2 cpm values of [^{3}H]thymidine incorporation for anti-CD3 activated NOD and BALB/c T cells represented by the highest supernatant dilution were 9,064±1,246 and 3,715±940, respectively. In *E*, BALB/c and NOD mice thymocytes were activated by plate-bound anti-CD3 in the absence or presence of soluble anti-CD28 (1/400 dilution of hybridoma ascites). Parallel cultures activated by anti-CD3 plus anti-CD28 were supplemented with anti-IL-2 (40 μg/ml), anti-IL-4 (40 μg/ml), or both mAbs. Supernatants from similar cultures were assayed for their IL-2 (*F*) and IL-4 (*G*) activities, as above. Percent inhibition of CD28 costimulation of thymocyte proliferation achieved by anti–IL-2 or anti–IL-4 are indicated above the corresponding bars. In *C–G*, the results of triplicate cultures are expressed as the mean values±SD, and are representative of four different experiments.

costimulation of BALB/c thymocytes (Fig. 1 *E*). CD28 costimulation of proliferation is therefore partially dependent on IL-4 production by NOD thymocytes, but not by control BALB/c thymocytes. We also found that anti–IL-4 partially blocked (46% inhibition) CD28 costimulation of IL-2 production by NOD thymocytes, but did not affect CD28 costimulation of IL-2 production by BALB/c thymocytes (Fig. 1 *F*). In contrast, anti–IL-2 inhibited almost completely (85% inhibition) the amount of CD28 costimulation of IL-4 production by NOD thymocytes, but did not block CD28 costimulation of IL-4 production by BALB/c thymocytes (Fig. 1 *G*). Thus, restoration of NOD thymocyte responsiveness by CD28 costimulation is dependent partially on enhanced IL-4 and IL-2 production.

Defective T cell activation in NOD mice (12, 39, 54) may account for the functional inactivation of regulatory peripheral Th2-like cells and lack of tolerance to pancreatic β cell antigens in NOD mice (3, 55, 56). Exogenous IL-4 prevents the onset of IDDM in NOD mice; this prevention is associated with the restoration of T cell proliferative responsiveness and augmentation of IL-2 production in vitro (13). Our data presented here suggest that NOD T cell proliferative responsiveness can be restored by CD28-mediated costimulation via a mechanism that is partially, if not primarily, dependent on the enhancement of IL-2 and IL-4 production, respectively.

CD28 costimulation prevents destructive insulitis and IDDM in NOD mice. Our result that IL-4 treatment of NOD mice prevents insulitis and IDDM (13), and finding in this report that CD28 costimulation markedly enhances IL-4 production by NOD T cells, prompted us to investigate whether CD28-mediated costimulation prevents insulitis and IDDM in NOD mice. We found that anti-CD28 treatment of NOD mice during the inductive phase (2–4 wk of age) of IDDM development prevents destructive insulitis (Fig. 2) and completely protects against IDDM (Fig. 3 *A*). Note that in 25-wk-old anti-CD28 treated NOD mice (Fig. 2 *B*), the percentage (19%) of islets displaying severe insulitis (insulitis score = 3) was considerably lower than that observed (46%) in control treated mice. Anti-CD28 treated animals still possessed 22% normal healthy

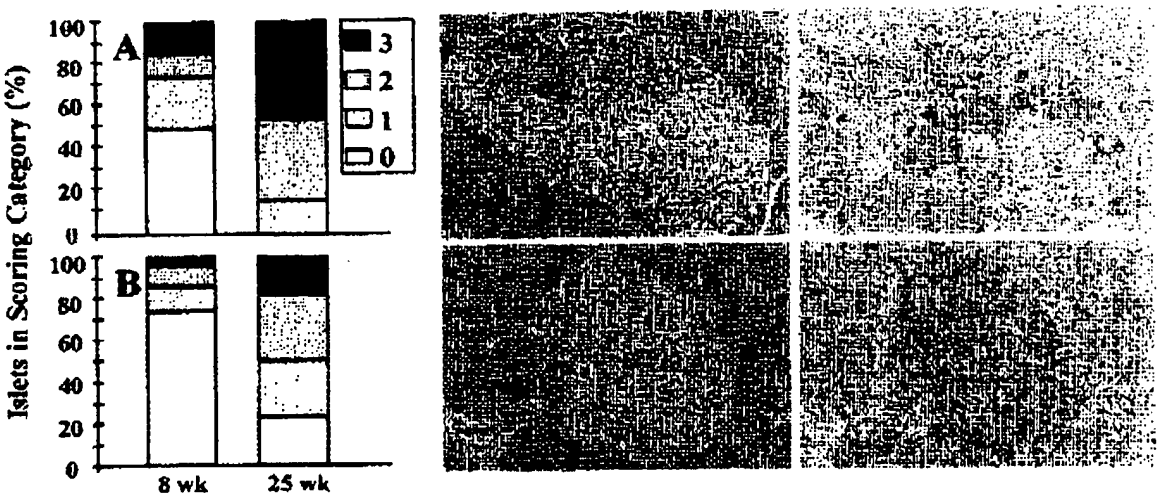

Figure 2. Anti-CD28 treatment prevents destructive insulitis in female NOD mice. After hematoxylin and eosin staining, a minimum of 20 islets from each NOD mouse were observed, and the degree of mononuclear cell infiltration was scored independently by two observers as follows: 0, normal; 1, periinsulitis (mononuclear cells surrounding islets and ducts but not infiltrating the architecture); 2, moderate insulitis (mononuclear cells infiltrating < 50% of the islet architecture); and 3, severe insulitis (> 50% of the islet tissue infiltrated by lymphocytes and/or loss of islet architecture). (*A*) 8- and 25-wk-old NOD mice ($n \geq 5$) injected with control hamster IgG. (*B*) 8-and 25-wk-old NOD mice ($n \geq 5$) injected with anti-CD28 mAb. Representative islets stained with hematoxylin and eosin (*photo left*) or insulin (*photo right*) are also presented. Note the destructive insulitis accompanied by a reduction in the number of insulin-producing cells in a representative islet from control IgG–treated mice at 8 wk of age (*A, photo left and right*). Note also the nondestructive periinsulitis in a representative islet from anti-CD28–treated mice at 8 wk of age (*B, photo left and right*).

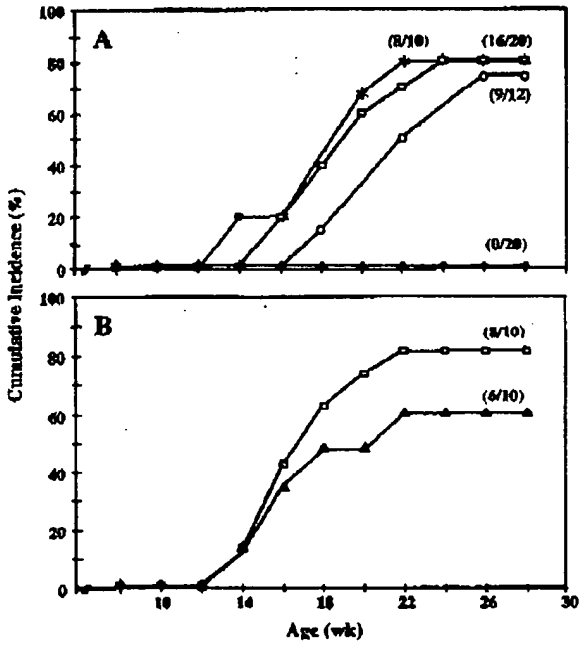

Figure 3. Anti-CD28 mAb treatment prevents IDDM in female NOD mice, and this protection is abrogated by anti IL 4 mAb treatment. (*A*) 20 female NOD prediabetic mice (randomized from five different litters) were injected three times weekly from two to four wk of age with 50 μg of either the 37.51 anti-CD28 mAb or control hamster IgG, and then boosted at six, seven, and eight wk of age. In addition, 10–12 mice (randomized from three to four different litters) were treated with anti-CD28 plus either the 11B11 anti–IL-4 mAb or control rat IgG, or 11B11 anti–IL-4 mAb alone. Control rat IgG treatment results are not shown, as they were similar to those obtained with control hamster IgG treatment. (*B*) Another group of ten females (randomized from three different litters) were similarly treated from five to seven wk of age. Mice were screened weekly for the presence of hyperglycemia (BGL > 11.1 mmol/liter) starting at 8 wk of age. Diabetes was diagnosed when mice were hyperglycemic for two consecutive readings. *Open squares*, control; *closed triangles*, anti-CD28; *asterisks*, anti–IL-4; *open circles*, anti-CD28 + anti–IL-4.

islets (insulitis score = 0), while normal islets were not present in the control animals. In contrast, when anti-CD28 mAb was administered together with anti-IL-4 mAb, the protective effect of anti-CD28 mAb was abrogated (75% of treated mice became diabetic at 25 wk) (Fig. 3 *A*). This result suggests that anti-CD28 mAb is an effective stimulator of IL-4 production by NOD T cells, and that the IL-4 secreted as a consequence of this stimulation is neutralized by anti–IL-4. Also note that NOD mice that received anti–IL-4 mAb alone displayed a similar incidence of IDDM as control IgG-treated mice. This observation is consistent with a report of the effect of treating NOD mice with various anticytokine mAbs, in which anti–IFN-γ was found to protect against IDDM while neither anti–IL-4, anti–IL-5, nor anti–IL-10 administration had any significant effect (57). Collectively, these results indicate that IL-4

plays an important role as a mediator of the anti-CD28 mediated protective effect.

Interestingly, when anti-CD28 treament was initiated after the onset of insulitis at 5 wk of age, significantly less protection from insulitis (data not shown) and IDDM (Fig. 3 *B*) was observed. This result emphasizes the age dependency of successful immune intervention in NOD mice (3, 28, 33). It also indicates that CD28 mAb administration may represent a form of immunostimulation of NOD T cells that effectively protects against IDDM, particularly when anti-CD28 treatment is administered during the inductive phase of the disease. Indeed, the same mAb purified from supernatants of the 37.51 B cell hybridoma was recently demonstrated to function by activation of CD28 signaling in vivo (58, 59).

Our observations in Figs. 2 and 3 are consistent with the recent report that disruption of the CD28/B7 pathway early in either CD28-deficient or CTLA4Ig transgenic NOD mice promotes the development and progression of IDDM (33). Together, these two sets of findings indicate that activation of NOD T cells by the CD28/B7 pathway is required to protect NOD mice from destructive insulitis and the onset of IDDM. Our data suggest that the anti-CD28 mAb used to treat NOD mice may prevent IDDM by activating the CD28 signaling pathway in NOD T cells rather than by blocking the interaction between CD28 and B7. Alternatively, we cannot formally rule out the possibility that anti-CD28 blocks CD28-B7-2 interaction, enabling a higher avidity interaction between CTLA4 and B7-1 to occur. The latter interaction may downregulate T-APC interaction and prevent IDDM in NOD mice (60). The age-dependent cytokine profiles (shown below) observed in thymocytes and peripheral T cells throughout the period of anti-CD28 treatment of NOD mice, viz. the high levels of IL-4 secretion compared with the decreasing levels of IFN-γ, however, also support the idea that anti-CD28–induced protection from IDDM is mediated by a polarized increase in Th2-like activity rather than a decrease in Th1-like activity.

Anti-CD28 treatment elicits the expansion and survival of Th2 cells. Prevention of IDDM by CD28 costimulation may be mediated by the activation of a subset of $CD4^+$ regulatory T cells that confer protection against IDDM. This subset of $CD4^+$ regulatory T cells may be hyporesponsive in NOD mice, and may not receive a sufficient amount of the CD28/B7 costimulatory signal required for clonal expansion and effector function in NOD mice (61). It has been proposed that precursor $CD4^+$ Th2 cells require a strong initial T cell stimulation, and that the amount of IL-4 produced is proportional to the magnitude of the initial T cell stimulation. In the absence of CD28 costimulation, IL-4 production remains below the threshold required for optimal development of Th2 cells (19, 20, 62). It is of interest that B7-1 and B7-2 ligation of CD28 mediate distinct outcomes in $CD4^+$ T cells. B7-2 costimulation signals naive T cells to become IL-4–producing T cells, and thereby directs an immune response towards Th0 and Th2 cells (26, 27, 63). B7-1 costimulation seems to be a more neutral differentiative signal, and initiates development of both Th1 and Th0/Th2 cells. Presumably, B7-2 plays a dominant role in production of IL-4 because of its early expression during T cell activation (20, 26). Thus, an insufficient or inappropriate signal resulting from a CD28/B7-2 interaction may be delivered to a subset of regulatory $CD4^+$ T cells in NOD mice, and this subset may not differentiate properly into functional IL-4 producing Th2 cells.

We tested this hypothesis by analyzing whether anti-CD28

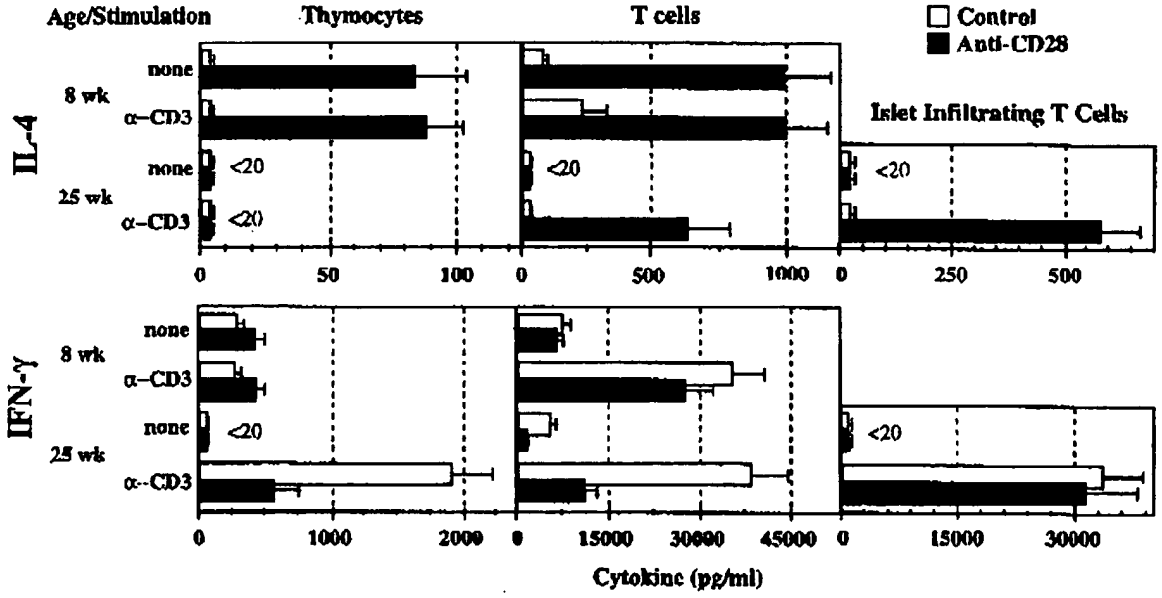

Figure 4. Anti-CD28 mAb treatment induces IL-4 production in NOD mice. Thymocytes, splenic T cells, and islet infiltrating cells (10^6/ml) were pooled from at least three age-matched NOD mice at various times after treatment with anti-CD28 mAb or control Ig, and were then stimulated with the 145-2C11 anti-CD3ε mAb (plate-bound, 1/500 ascites dilution). After either 72 (thymocytes) or 48 h (T cells and islet infiltrating cells) of culture, the concentrations of IFN-γ and IL-4 in cell supernatants from triplicate cultures were determined by ELISA. Values shown are the mean±SEM of three separate experiments.

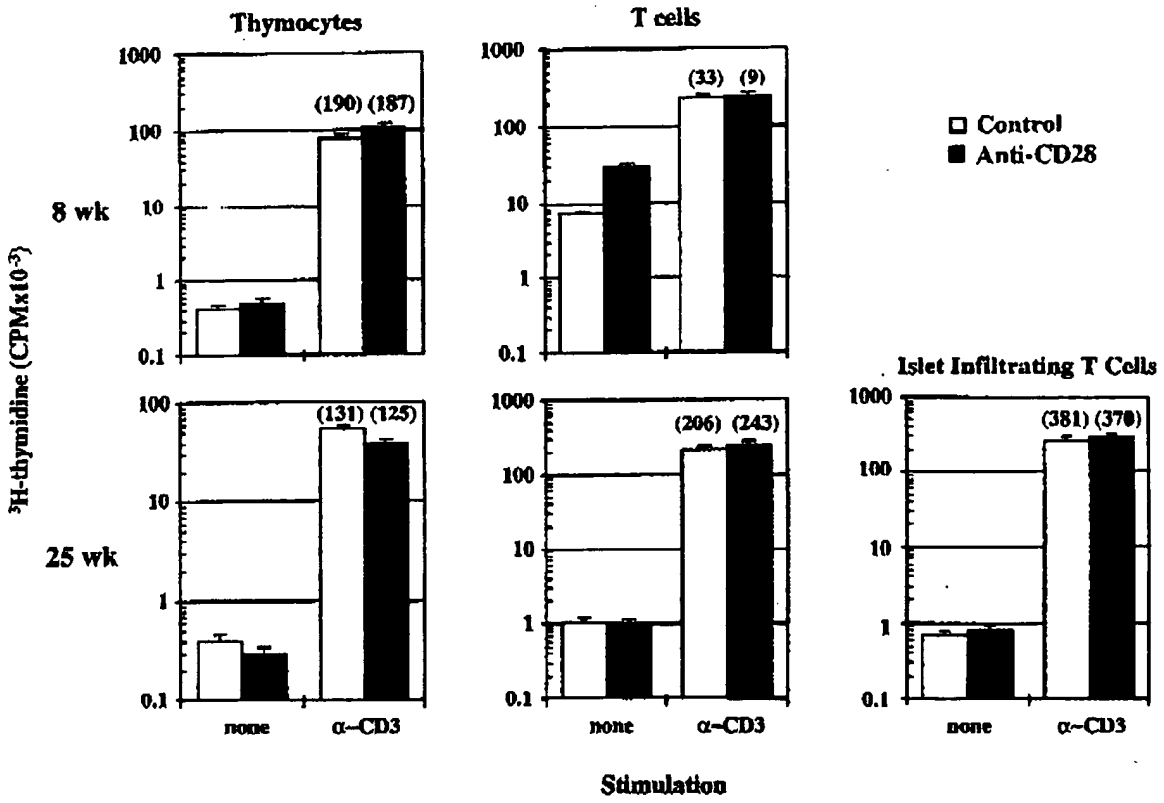

Figure 5. Anti-CD28 mAb administration in vivo enhances basal splenic T cell proliferative responses but not T cell proliferation in response to anti-CD3. Thymocytes, T cells and islet infiltrating cells (2×10^5/well) from 8- and 25-wk-old NOD mice ($n \geq 3$) injected with either anti CD28 mAb or control hamster Ig were cultured in triplicate wells in the presence of the plate-bound 145-2C11 anti-CD3ε mAb (1/500 ascites) for 48 (T cells, infiltrating cells) or 72 h (thymocytes). Cell proliferation was determined by [^{3}H]thymidine incorporation. Values (mean cpm±SD) shown are representative of three separate experiments. Stimulation indices were calculated as the ratio of average cpm of anti-CD3 stimulated cultures/average cpm of control cultures, and are shown in parentheses.

mAb treatment of NOD mice provides the costimulation required for the expansion of and cytokine production by regulatory IL-4–producing Th2-like cells. Fig. 4 shows that anti-CD3–stimulated (in vitro) NOD thymocytes obtained at 8 wk, peripheral splenic T cells obtained at 8 wk and 25 wk, and islet-infiltrating T cells examined at 25 wk of age produce significantly higher levels of IL-4 when compared with the same subpopulations of cells isolated from control mice treated with a hamster Ig. Interestingly, shortly after termination of treatment with anti-CD28 mAb, thymic and splenic T cells showed a higher basal (no stimulation) production of IL-4 compared to cells obtained from age-matched (8-wk-old) control mice. With the exception of a 4.3-fold higher splenic T cell basal response in 8-wk-old anti-CD28 treated mice, no differences were detected between the proliferative responses of thymocyte, splenic T cells, and islet-infiltrating cells from 8 and 25-wk-old anti-CD28–treated NOD mice and those of the age-matched controls (Fig. 5). The increase in basal T cell proliferation and IL-4 production may reflect the preferential costimulation of Th2 cells by anti-CD28 treatment in vivo. Indeed, we found that anti-CD28 treatment in vivo leads to an increased production of IgG1 (which reflects increased IL-4 production by T cells) rather than IgG2a anti-GAD67 antibodies (Fig. 6 *B*). Moreover, the total number of splenic lymphocytes was increased about 1.9-fold at 8 wk of age and 1.7-fold at 25 wk of age in anti-CD28–treated NOD mice relative to that of control-treated mice (our unpublished data). These findings, together with our observation that anti–IL-4 treatment in vivo blocks the anti-CD28–induced protection from IDDM, support the idea that anti-CD28 treatment elicits the expansion and survival of IL-4–producing Th2 cells in NOD mice.

Anti-CD28 treatment did not significantly alter the level of IFN-γ secretion by T cells from 8-wk-old NOD mice when compared with that observed in age-matched control mice. Levels of IFN-γ secretion by thymocytes and splenic T cells from 25-wk-old anti-CD28–treated NOD mice, however, were markedly reduced in comparison to those levels detected in control mice. These data demonstrate long-term downregulation of Th1 cell function, which may arise from the preferential activation of Th2 cells induced by CD28 costimulation during the inductive phase of the autoimmune process. The downregulation and/or functional deviation of Th1 cells towards a Th2 cell phenotype by IL-4 is more effective than and dominant over the inhibition of Th2 cell function by IL-12 (64–66). In-

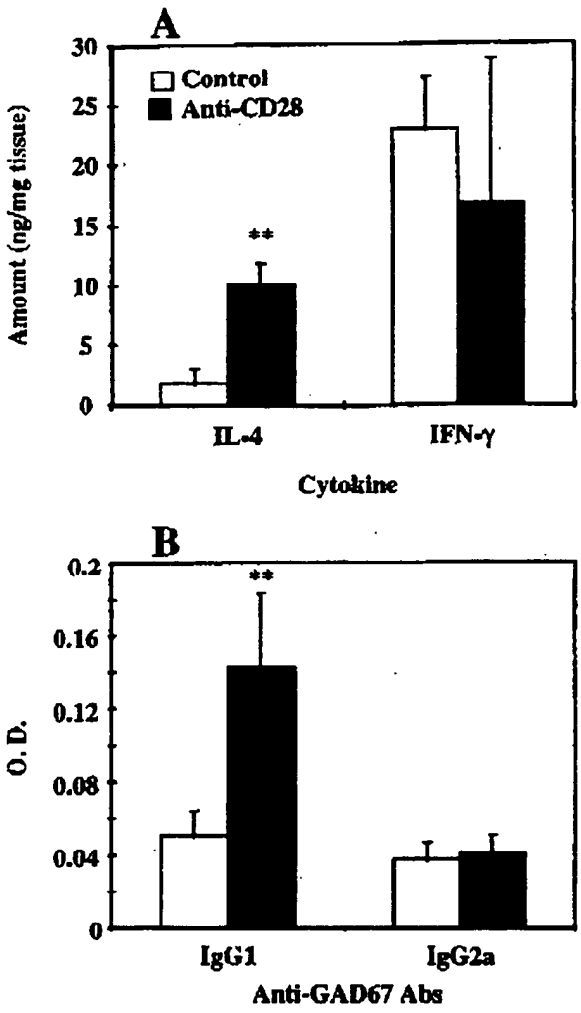

Figure 6. Intrapancreatic content of IL-4 and serum concentration of anti-GAD_{67} antibodies of the IgG1 isotype is enhanced after CD28 costimulation in vivo. (*A*) NOD mice treated with either anti-CD28 mAb ($n = 7$) or control hamster Ig ($n = 5$) were killed at 25 wk of age, and the intrapancreatic IL-4 and IFN-γ concentrations were determined by ELISA. Values are expressed as mean ng/mg of total protein extracted from pancreatic tissue samples. Comparison between means was performed by Student's *t* test, and a *P* value of < 0.05 was chosen as the level of significance (**$P < 0.001$). (*B*) Sera were collected and analyzed by ELISA for the presence of anti-GAD_{67} antibodies of the IgG1 and IgG2a isotypes as described in Methods.

deed, when the development of a Th2 response is mediated by strong CD28 costimulation, the Th2 response is less sensitive to the inhibitory effects of IL-12 (67). Thus, our results agree with reports that IFN-γ–secreting Th1 cells potentiate the effector phase of insulitis, IFN-γ is directly involved in β cell destruction (68–71), and the early differentiation of naive T cells into Th2 cells is dependent on CD28 signaling (33, 36).

CD28 ligation promotes production of Th2 cytokines by naive murine T cells in an IL-4–dependent manner (72). Studies of the role of CD28 and B7 in the differentiation of Th1 and Th2 cells in a mouse model of Leishmania infection, however, show that CD28, although important, is not an absolute requirement for generation of a Th2 response (73). This result suggests that the mouse strain genetic background may contribute to Th1/Th2 phenotype polarization as much as costimulation. Nonetheless, our results of modulation of Th1/Th2 phenotype in NOD mice, together with the results of others obtained in either additional strains of autoimmune mice or in analyses of antigen-specific T cell responses in vitro (33, 36, 72), illustrate a critical role for CD28 costimulation in Th1/Th2 phenotype regulation. It is possible that the discrepancy between the results obtained in Leishmania-infected mice and those observed in autoimmune mice may be attributable to the high magnitude of the activation signal(s) delivered to T cells induced by Leishmania infection. The strength of this signal(s) may overcome any genetic deficiencies in T cell development as a result of compensatory mechanisms such as upregulation of other costimulatory surface receptors on T cells and/or the production of potent cytokines (49, 67, 73), including IL-6, which has an important role in the control of Th2 cell differentiation (74).

Although anti-CD28 mAb treatment protects from IDDM, this treatment still allows for development of a nondestructive periinsulitis, and therefore does not interfere with migration of diabetogenic T cells to the pancreatic islets. Rather, anti-CD28 treatment appears to induce regulatory T cells in the pancreas to suppress islet β cell destruction and progression to overt IDDM. Evidence in support of this notion is derived from assays of secretion of IL-4 and IFN-γ by infiltrating cells from mice treated with anti-CD28 or control Ig (Fig. 4) and from analyses of the levels of expression of these cytokines in the pancreas of anti-CD28 treated NOD mice at 25 wk of age (Fig. 6). Note that intrapancreatic expression of IL-4 is significantly higher in anti-CD28 mAb–treated mice, whereas the expression of IFN-γ remains essentially unaltered in these mice. Committed autoreactive cells, including Th1 cells, may accumulate in pancreatic islets, but the function of IL-4 predominates to inhibit IFN-γ–mediated β cell damage.

Results of comparative FACS analyses of the phenotype and surface expression of various cell adhesion molecules and T cell activation markers in anti-CD28 mAb–treated and control IgG–treated NOD mice at 8–25 wk of age are consistent with our observation that anti-CD28 treatment does not block the migration of T cells to pancreatic islets. We found that the levels of expression of LFA-1, L-selectin, CD44, CD-69, ICAM-1, CD28, and B7-2 on splenic T cells did not differ significantly between anti-CD28–treated and control-treated NOD mice (our unpublished data). Similarly, no significant differences in expression of B7-1 and B7-2 were detected on splenic APCs (including B cells) from anti-CD28 versus control-treated NOD mice. In addition, the level of expression of CD28 on splenic T cells and of B7-1 and B7-2 on splenic APCs were the same in the NOD and control strains of mice. Lastly, the T ($CD3^+$):B ($CD19^+$) and CD4:CD8 T cell ratios in NOD mice were not altered by anti-CD28 treatment.

When splenic T cells from NOD mice (25 wk of age) were transferred into NOD.Scid recipients, the transfer of IDDM was either prevented or significantly delayed if recipient mice received T cells from anti-CD28–treated donors (Fig. 7). All (5/5) of the mice transferred with T cells from control IgG–

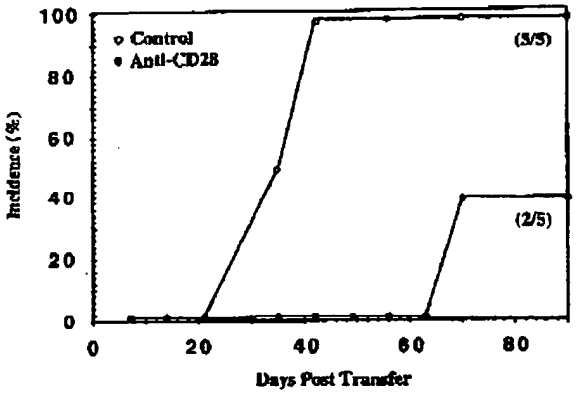

Figure 7. Adoptive transfer of T cells from anti-CD28 treated mice prevents/delays onset of IDDM in NOD.Scid recipients. Splenic T cells (10^7) from prediabetic female NOD mice previously treated or untreated with anti-CD28 mAb were injected into female NOD.Scid mice (n = 5/group). The recipients were followed for a maximum of 12 wk after transfer, and BGL were monitored weekly.

treated mice became diabetic between 35–40 d after transfer, while only 2/5 of the mice transferred with T cells from anti-CD28 treated animals developed diabetes by 90 d after transfer. These results are compatible with a previous report that Th1 cells, but not Th2 cells, can transfer IDDM to NOD.Scid recipients (11), and suggest that anti-CD28 mAb treatment activates regulatory Th2 that remain functional and prevent/reduce IDDM over a prolonged period. This effect may arise from the ability of CD28 ligation to sustain the proliferative response and enhance long-term survival of T cells by delivering a signal that protects from apoptosis through upregulation of survival factors such as Bcl-x_L (75–77).

Concluding remarks. As previously suggested (78), CD28/B7 interactions may differentially regulate Th1/Th2 responses depending on the phase of the disease at which immune intervention is initiated. Blockade of CD28/B7 during the early phase of onset of an autoimmune disease preferentially blocks differentiation of the Th2 cell lineage and exacerbates the disease. Experiments conducted with CD28-deficient mice demonstrate that CD28 expression is required for predominant Th2 cell responses in an autoimmune environment (32, 33). Compatible with these findings, our results suggest that the activation of the CD28/B7 pathway during the early phase of onset of insulitis in NOD mice may stabilize a protective Th2-mediated environment in pancreatic islets. This type of environment appears to protect against a destructive insulitis and progression to IDDM. In contrast to other mAbs that protect NOD mice from IDDM, such as anti-CD4 and anti-CD3 mAbs (79, 80) the anti-CD28 mAb we used in this study appears to selectively activate a subset of regulatory Th2 cells by stimulating T cell clonal expansion in vivo, presumably by activating CD28 signaling and augmenting IL-2 and IL-4 secretion. Anti-CD28 mAb treatment during the early inductive phase of diabetogenesis and much before the onset of disease therefore represents an effective means of immunostimulation. This treatment affords a promising type of immunotherapy for the IDDM prevention in individuals at high risk for the disease.

Our data are compatible with the notions that (*a*) Th2 cells require a higher threshold of activation than Th1 cells; (*b*) in NOD mice, Th2 cells are deficient in their ability to receive this CD28-dependent signal from interacting APCs, and (*c*) this CD28 signal is requisite for activation of IL-4–producing NOD Th2 cells and protection from IDDM. We cannot, however, rule out the alternate possibility that NOD APCs may not provide an appropriate or sufficient CD28 costimulation signal during the inductive phase of IDDM development. Further experimentation is required to discern between these possibilities. In conclusion, our results indicate that augmenting costimulation at an early age can completely prevent development of a spontaneous organ-specific autoimmune disease.

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Insulin-Dependent Diabetes Mellitus as an Autoimmune Disease

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I. Introduction

DIABETES mellitus is simply defined on the basis of hyperglycemia. It is, however, a highly heterogeneous disease. A major advance was made in the late 1960s when insulin-dependent diabetes mellitus (IDDM, type 1) was distinguished from non-insulin-dependent diabetes mellitus (NIDDM, type 2). Another milestone was the realization in the 1970s that in most cases IDDM has, presumably, an autoimmune origin (1–4). This offered new clues to the etiology and elicited hopes of immunoprevention, which is still the ultimate goal of research in the immunology of IDDM.

This review will attempt to cover the major pending questions on the origin of the autoimmune process that leads to IDDM and will discuss in some depth genetic predisposition and environmental factors, the interaction of which creates the conditions required for disease onset. This will be followed by a characterization of the anti-β-cell immun response and the mechanisms by which the β-cell lesion is induced. Also discussed will be how physiological tolerance to self-antigens of β-cells is lost in diabetic subjects, as it is the pathogenic event underlying T cell-mediated β-cell aggression. The review will conclude with present and potential clinical applications of these concepts, which have already changed the face of diabetology and will continue to gain momentum. Animal models of the disease will be presented first and will figure strongly throughout this review, inasmuch as they have provided exceptional means for genetic and immunological manipulations inaccessible in man.

II. Animal Models f IDDM

A broad spectrum of animal models of IDDM have become available over the last 10 yr. They comprise spontaneous models, in which disease develops unprovoked, and experimental models induced by various types of intervention.

A. Spontaneous models

Two major models of IDDM are used: the nonobese diabetic (NOD) mouse and the Bio Breeding (BB) rat, which develop a disease very similar, by most evaluable criteria, to human IDDM.

1. The NOD mouse. The NOD mouse was discovered in Japan in the late 1970s (5). It was inbred, distributed worldwide, and used to establish numerous colonies. These colonies differ widely in the frequency and the age of onset of IDDM (6), owing to multiple environmental factors (see below). Diabetes usually appears between 4 and 6 months of age, much more frequently in females than in males. Clinical diabetes is preceded by infiltration of the pancreatic islets by mononuclear cells (insulitis), which occurs at about 1 month of age in both sexes. In addition to diabetes, NOD mice present thyroiditis (7), sialitis, and, late in life, autoimmune hemolytic anemia (8). Extrapancreatic autoimmune manifestations, including thyroiditis, are also found in a subset of human diabetics with female preponderance (sometimes called type 1b). Recently, interesting new experimental tools have been constructed. They include the NOD/nude mouse, where the nude (athymic) genotype has been introduced by repeated backcrosses in the NOD mouse background (9), and the NOD/scid mouse, in which a mutant gene encoding a defect common to both site-specific DNA recombinational and DNA repair pathways has been introduced into the NOD genome, leading to a severe combined immunodeficiency (10). These models can be used to perform unique experiments of cell transfer without interference from the (deficient) recipient immune system. Also noteworthy is a model of accelerated diabetes induced by cyclophosphamide, an alkylating agent widely used as an immunosuppressive drug. Two injections of 200 mg/kg at a 14-day interval induce diabetes in most male and female mice within 2 to 3 weeks (11, 12) through a mechanism probably involving elimination of regulatory T cells (discussed below).

2. The BB rat. The BB rat was initially developed in Canada in the early 1970s (13). At about 4 months of age it develops severe diabetes, preceded, as in the NOD mouse, by insulitis. A particular feature of the BB rat is the presence, early in life, of major lymphocytopenia (14), involving a particular lymphocyte subset characterized by the RT6 antigen (15). Diabetes is usually associated with thyroiditis in this model. Not all BB rats develop diabetes: a subset of BB rats representing a genetic drift are diabetes resistant (DR-BB).

B. Experimentally induced diabetes

At variance with most autoimmune diseases, in which the target autoantigens are known, we as yet have no experimental model of diabetes induced by administration of the target β-cell autoantigen incorporated in adjuvant, with the exception of transient diabetes induced by a peptide derived from a candidat target autoantigen, heat shock protein 60 (16, 17). Fortunately, numerous other experimental models are available.

1. Chemically induced diabetes. Streptozotocin (STZ)-induced diabetes. β-Cell destruction can be achieved by administering high doses of β-cell-selective toxic agents such as STZ (18) and alloxan (19). Repeated administration of STZ at low, subdiabetogenic doses also causes diabetes preceded by insulitis (20). Such low dose STZ-induced diabetes appears to be immunologically mediated, as indicated by resistance of athymic mice (21) and prevention by immunosuppressive agents (20) even if some intriguing data have recently been reported showing induction of the low-dose STZ diabetes in NOD-scid/scid mice in the absence of functional lymphocytes (21a). The mechanisms of insulitis and diabetes appear to relate to STZ-induced changes in islet immunogenicity: insulitis only appears on islets grafted in STZ-treated mice if grafting is performed before STZ administration or if the islets are first exposed to STZ *in vitro* (22). The mechanisms of these changes are not fully understood but might be related to the induction by STZ of increased expression of class II molecules of the major histocompatibility complex (MHC) on β-cells. This increased expression has been directly visualized (23), and low-dose STZ-induced diabetes is prevented by anti-interferon-γ (IFNγ) antibody therapy (24), which is known to inhibit MHC molecule expression. The relevance of this mechanism to human IDDM pathogenesis will be discussed later, but it is interesting to note here that NOD mice are susceptible to lower repeated STZ doses than conventional strains with the highest STZ sensitivity (25, 26), pointing to the possible role of toxic environmental factors in genetically predisposed individuals.

2. Immunomanipulation. Thymectomy performed within 2 days after birth can induce a flourishing state of autoimmunity in mice (27). Whether the emergence of autoreactive clones is due to elimination of the censor function of the thymus (negative selection of autoreactive clones) or to the loss of suppressor function is still being debated (27). Similarly, insulitis and diabetes (associated with thyroiditis) can be induced in normal non-autoimmune adult rats by combining adult thymectomy and sublethal irradiation (28, 29) or in athymic rats by transfer of normal spleen cells (30). The disease can be prevented by administration of CD4+RT6+ T cells derived from normal rats (28) or facilitated in the adoptive transfer model by prior depletion of RT6+ cells *in vivo* (30), suggesting that in both models diabetes is due to the elimination of a RT6+ T cell subset with suppressor function. It is interesting to note the paradox between these models in which thymectomy promotes diabetes and the observation, discussed later, that neonatal thymectomy prevents the onset of diabetes in NOD mice and BB rats. One may presume that in the latter case thymectomy prevents the differentiation of effector T cells (perhaps together with that of helper T cells) while in the former, where thymectomy is slightly delayed, there is only inhibition of suppressor T cell differentiation.

3. Transgenic mice. Selective expression of various transgenes in β-cells can be induced by c upling them to the insulin gene promoter. This strategy has been applied successfully to a number of models, leading to the induction of insulitis and/or diabetes. Insulitis, the hallmark of immunologically mediated diabetes, can be induced in mice transgenic for the simian virus SV40 T antigen gene when the transgene is expressed in β-cells late in ontogeny (after thymic negative selection has taken place) (31). Insulitis is the consequence of an anti-T antigen T cell-mediated response. Interestingly, when the T antigen is expressed earlier in ontogeny, mice are tolerant to the antigen and do not become diabetic (but they may then develop insulinoma). Similar results can be obtained with the IFNγ gene, which probably operates by enhancing the expression of class II MHC molecules in β-cells (32). Diabetes in such transgenic mice is of an autoimmune nature, since the disease is transferred to normal syngeneic islets grafted into the transgenic mice, and lymphoid cells from the transgenic mice are cytotoxic to normal isl ts *in vitro* (33). Similar but less clear-cut data have been reported with IFNα (34), tumor necrosis factor-α (TNFα) (35, 36), and interleukin (IL)-10 (37). Interestingly, in the two latter cases insulitis occurred but diabetes did not (*i.e.* there was no β-cell lysis).

Another approach consists of expressing various genes, notably viral genes, early enough in development to prevent anti-β-cell sensitization and then attempting to provoke IDDM either by infecting the mice with the corresponding virus or by hybridizing them with other transgenic mice expressing the genes for T cell receptors (TCR) specific to the transgene-encoded antigen. Oldstone *et al.* (38) showed that transgenic mice expressing the gene of the murine lymphocytic choriomeningitis virus (LCMV) glycoprotein became diabetic after infection with LCMV, due to destruction by antigen-specific cytotoxic T cells of β-cells expressing the viral antigen. This shows that selective expression of the viral antigen in β-cells (and presumably not in the thymus) early in development does not lead to tolerance toward this antigen, since it would have then prevented sensitization in the adult. Insulitis and diabetes have been observed in the absence of viral infection in transgenic mice expressing the influenza virus hemagglutinin in β-cells (39). One should note, however, that in a similar model Lo *et al.* (40) failed to induce diabetes in transgenic mice expressing the influenza virus hemagglutinin in β-cells even after infection with hemagglutinin-expressing viruses.

The double transgenic strategy has been used successfully for the influenza virus hemagglutinin (40a) and the glycoprotein of LCMV (41). It is important to note that infection by the virus was necessary to obtain diabetes in the case of LCMV glycoprotein, suggesting that in certain experimental conditions nontolerant T cells may ignore their target antigens expressed in β-cells. Viral infection may then stimulate the recognition of the antigen and T cell activation, indicating that overcoming ignorance may require T cell preactivation. Virus infection was not required to obtain diabetes in the influenza hemagglutinin model. This difference suggests that, depending on the transgenic mice utilized (*i.e.* MHC and non-MHC genotype, MHC class I or class II restriction, environmental factors, etc.), coexpression of the target antigen in β-cells and the corresponding TCR is sufficient for diabetes to occur in the absence of T cell activation. We shall see below that NOD mice transgenic for the TCR of diabetogenic T cell clones develop accelerated diabetes. However, it should be emphasized that coexistence of the antigen and the specific TCR does not necessarily lead to elimination or activation of these T cells suggests that, in these settings, T cells may "ignore" their target antigen.

Diabetes can also occur in transgenic mice expressing MHC class I (42) or class II (43–44) genes in β-cells; however, in this case diabetes is not due to an immune reaction (*i.e.* there is no insulitis) but rather to β-cell functional alterations due to overexpression of multiple copies of the MHC molecules. Indeed, β-cell expression of smaller amounts of MHC molecules does not induce diabetes (45), and expression f non-MHC molecules such as calmodulin can induce a similar type of nonimmune IDDM (46).

TABLE 1. Transgenic mice for the study of IDDM

Immune diabetes or insulitis.		
Single transgenics (transgene coupled to insulin promoter)		
SV40-T antigen (31)		
IFN-γ (32, 33, 34)		
TNF (35, 36)		
IL-10 (37)		
LCMV glycoprotein + virus infection (38, 242)		
Influenza virus hemagglutinin (39)		
Double transgenics		
Influenza virus hemagglutinin (β-cells) + TCR (40a)		
LCMV + TCR + virus infection (β-cells) (41)		
Nonimmune IDDM (without insulitis)		
MHC class I (42)		
MHC class II (43, 44)		
Calmodulin (46)		
Transgenic NOD mice (protection from insulitis and/or diabetes)		
I-A	$I\text{-}A^d$	(257)
	$I\text{-}A^k$	(53, 54, 256)
	Aα (Pro 56)	(55)
I-E	E^{C57BL}	(56)
	$E\alpha^d$	(55)
	$E\alpha^k$	(57)
L^d		(58)

C. Lessons from animal models

These animal models have enabled us to make remarkable progress over the last few years in understanding the pathogenesis of IDDM. They have been used for the transfer experiments necessary to prove the autoimmune nature of the disease and have allowed the production of islet-specific T cell clones and enabled fine analysis of MHC and non-MHC diabetes predisposing genes. Finally, they have allowed the evaluation of the various immunointervention procedures to be potentially used in man.

It is essential to bear in mind, h wever, that IDDM is a heterogeneous disease (see *Section IX.A*) and that each animal model represents, at best, the counterpart of an individual

human case reproduced in multiple copies. In addition, it should be remembered that most xperimentally induced models corresp nd to highly artificial situations far from the conditions in which spontaneous disease develops.

III. Genetics of IDDM

A. Introduction: familial transmission of the disease

IDDM has long been known as a hereditary disease on the basis of the relatively high rate of familial transmission: the risk of becoming diabetic is approximately 7% for a sibling and 6% for a child of a diabetic (47). The disease concordance rate is approximately 35–40% in identical twins (47, 48) but penetrance of genetic factors evaluated from the identical twin concordance rate is probably less than 40% for the f llowing reasons: 1) twins share more environmental factors than unrelated individuals, 2) there is a tendency for disease-concordant identical twins to respond more to population calls than nonconcordant twins, and 3) a significant percentage of twins carrying the whole set of predisposing genes are both resistant to the disease.

B. Approaches to identifying IDDM predisposition genes

The above patterns of familial transmission, combined with data from animal models, indicate that the determinism of IDDM is polygenic and multifactorial. The search for predisposition genes is complex, especially as most if not all predisposition genes appear to be basically "normal" *i.e.* without mutations or deletions. A fortuitous combination of these genes, together with permissive or triggering environmental factors, provokes the disease. Each of these genes may be present in a large proportion of healthy subjects (notably the patient's nondiabetic relatives).

There are two distinct strategies for identifying IDDM predisposition genes. In the first, one selects candidate genes coding for presumed elements of the pathological process such as the T cell receptor, MHC molecules, β-cell autoantigens, insulin, and cytokines and seeks links between their polymorphism and the disease.

In the second approach (identical to that used for monogenic diseases), segregation of the disease or of one of its major traits (partial phenotype) and that of polymorphic markers distributed throughout the genome are studied in parallel in multiplex families. The most easily accessible and informative markers now available are microsatellites, *i.e.* simple sequence repeats whose length varies between individuals in an allele-stable fashion.

C. The role of the MHC

Given the major role of MHC molecules in antigen presentation to T cells, MHC genes are obvious candidate predispositi n genes f r IDDM (and all oth r autoimmune diseases), even if, in fact, their association with IDDM was discovered fortuitously (49, 50), before MHC restriction of antigen recognition by T cells was unraveled.

The role of the MHC in genetic predisposition to IDDM is predominant, as shown by the high disease concordance rate in HLA-identical siblings (~12%, and even 15-17% in DR3/4 heterozygotes) (47). It is also fully confirmed in murine models of the disease by segregation studies (51), by the absence of diabetes observed in congenic mice genetically identical to NOD mice except for the MHC (52), and by the prevention of the disease by introduction of various MHC transgenes differing from the NOD MHC, either class II (I-A) (53–55), I-E (55–57), or class I (58).

1. Animal models. Diabetes onset is closely dependent on the MHC in NOD mice at the level of the I-A locus (51, 59) in a dominant fashion (52). Sequence analysis of the I-A^{NOD} gene has shown that this allele has a serine residue at position 57 of the β-chain at variance with all common mouse strains that have an Asp at that position (60). However, absence of Asp at position 59 does not entirely explain the role of the MHC, since transgenic mice expressing I-A genes without an Asp at position 57 of the I-Aβ-chain can be protected from diabetes (54, 55). Also, NOD mice do not express genes of the other I locus, I-E, owing to a mutation of the Eα promoter region (59). NOD mice transgenic for I-E (55–57) are protected from the disease, an important finding suggesting a protective role of I-E genes even though I-E+ NOD mice obtained by backcrossing with I-E+ strains may develop diabetes (61). Segregation studies have also pointed to the major predisposing role of the MHC in the BB rat with partial dominance of the RT-1^u-allele (62). Interestingly, in RT-1^u × RT-1^b crosses, diabetes is associated with the u-allele, whereas thyroiditis is associated with the b-allele (63). It remains to be determined whether class II loci are exclusively involved in the MHC-associated predisposition to diabetes in these animal models (independently from the numerous non-MHC predisposing genes to be discussed below).

2. Human IDDM. IDDM is positively associated in Caucasians with two sets of alleles: 1) HLA A1, B8, DR3 DQB1*0201 and 2) DR4 DQB1*0302 (64–65). This association was initially shown by means of serological typing (49, 50) and has now been confirmed by direct genomic typing with polymerase chain reaction and hybridization using sequence-specific oligonucleotide probes (66, 67). The association holds for alleles on neighboring loci (haplotype) because of the tight linkage disequilibrium in the MHC. This is particularly true for the ancestral extended haplotype A1 B8 DR3 DQB1*0201 DQA1*0501, which comprises class III genes and the TAP2*0101 allele, making it difficult for this haplotype to determine the precise locus that predisposes to the disease. The case is clearer for DR4-DQB1*0302, where the DQ locus seems to be directly involved (64, 65) in keeping with the putative Ir gene function of class II genes (*i.e.* HLA class II molecules bind antigenic peptides and present them as a molecular complex to the TCR). Attention has been drawn to the nature of the residue at position 57 of the HLA DQβ-chain (absence of Asp in IDDM-predisposing alleles) (68). The Asp residue is much more rarely found in diabetics than in the general population and almost never in double copy (homozygous state). This observation is particularly interesting in view of th critical place of this residue in MHC-peptide interactions. The highest relative risk is ob-

served in DR3/4 heterozygotes, with a disease frequency higher than that predicted from the relative risks associated with individual alleles. It is not known whether this apparent synergy is due to the synergistic interaction between two independent HLA genes or to the creation of a hybrid molecule made of chains encoded by the two alleles (65). Such transcomplementation has been formally demonstrated (69) but its pathogenic role is uncertain.

Other MHC genes are associated with IDDM protection, their frequency being lower in diabetics than in the general population. This is the case for DR2 (63, 64, 66, 67) and for the TAP2*0201 allele (70, 71), which codes for a transporter of antigenic peptides to MHC class I molecules. Whether this latter association is intrinsic or relates to a linkage disequilibrium with class II alleles remains to be determined. It is important to continue investigations of the mechanisms of MHC-associated IDDM protection, which could include, in addition to defective peptide transport, peptide capture by the protective HLA molecules that prevents binding of the peptide to the predisposing HLA molecules and, thus, its effective presentation to T cells or the generation of suppressor cells of the TH2 type (63). Finally, note that analysis of the MHC-IDDM association is complicated by disease heterogeneity, notably in terms of age of onset (67) and ethnic origin (63–65).

D. Non-MHC genes

The involvement of non-MHC genes in the predisposition to IDDM is demonstrated by the above mentioned difference in the disease concordance rate in identical twins (35–40%) and HLA-identical siblings (~12%). The search for candidate non-MHC predisposing genes has so far been relatively unfruitful in human IDDM. Nonetheless, the insulin gene has been shown to be associated with IDDM (72–74), particularly in HLA DR4 subjects (73). In the same study, it was shown that the insulin gene effect was stronger in paternal meiosis, suggesting a role for maternal imprinting (72). However, the involvement of these two features (DR4 preference and paternal meiosis) was not confirmed in another study (74). It remains to be shown whether the association relates to the insulin gene itself, as suggested by a recent mapping study (75), or to a neighboring gene. Studies of the polymorphism of another logical candidate gene, TCR, have failed to provide clear-cut results (76–78).

Studies of the NOD mouse have been more fruitful. Segregation studies using microsatellites have led to the description of 12 non-MHC predisposition loci (Refs. 51 and 79–83 and Table 2), in addition to the major association with MHC loci on chromosome 17. One of the genes on chromosome 1 could be bcl2 (80), a proto-oncogene known to have anti-apoptosis functions. Delayed T cell apoptosis, directly demonstrated in NOD mice (84), could favor survival and activation of autoreactive T cells, in keeping with similar data obtained in MRL/l lupus mice showing a mutation of the FAS gene, known for its apoptosis function. One of the genes on chromosome 3 has been narrowed down to the IL-2 gene, which has a different sequence in NOD mice than in common mouse strains, including an insertion and a deletion of tandem repeat sequences that encode amino acid repeats in th mature protein (81). The other gene on chromosome 3 has been mapped to the gene coding for the higher affinity receptor for immunoglobulin G (83). The nature and expression of the other predisposition genes are unknown.

TABLE 2. Genes predisposing to IDDM

		References
NOD mouse		
MHC (ch 17)	I-A	51, 52, 59, 81
	I-E (absence of expression)	59
Ch 1	IL-1R	79
	bcl2	80
Ch 3	IL-2	81
	high affinity Fcγ receptor	51, 81–83
Ch 4		81
Ch 6		51, 81, 82
Ch 7		81
Ch 9		81
Ch 11 (early-onset cytoxan-induced diabetes)		51, 81
Ch 14		81
Ch 15		82
BB rats		
MHC ($RT1^u$)		61
Ch 4 lyp (lymphocytopenia)		85
Man		
MHC		64–68
A1 B8 DR3 DQB 201 DQA 501 TAP2-A		
DR4 DQB 302 DQA 301		
+ protection DR15(2) DQB 602 DQA 102		
Insulin		72–75

Studies in the BB rat have been less informative. They indicate, however, that lymphocytopenia is encoded by the autosomal gene lyp on chromosome 4, close to the neuropeptide Y gene (85).

E. Conclusions

Taken together, these data suggest the existence of stage-specific genetic control of IDDM. bcl2 And other genes could control an intrinsic nonantigen-specific anomaly of T cells, which could explain the initial mononuclear cell infiltration of the islets (periinsulitis) and other organs (*e.g.* sialitis), as well as the association with other autoimmune traits. The MHC would then play the central role in β-cell autoantigen recognition. Other genes are probably involved, such as those coding for immunoregulatory cells (that amplify the autoimmune reaction), notably cytokine genes (*e.g.* the mutant IL-2 gene mentioned above) and genes controlling β-cell sensitivity to the immune aggression. When these genes are identified, the problem will be to determine their relative contribution to genetic predisposition. It may turn out that all susceptibility genes (defined on the basis of segregation studies) are effectively involved in the pathogenic process, but that their contribution to increasing the relative risk may be highly variable; this will depend not only on the importance of their functional role but also on the frequency of the predisposing allele in the general population. The fairly high concordance rate between siblings, despite relatively low penetrance, argues for a small number of major predis-

positi n genes (MHC plus perhaps two or thr e non-MHC genes). This does not, however, rule out the involvement of a multitude of minor genes with an accessory pathogenic role (not mandatory), present in a large fraction of the general population or "used" in a very limited number of patients (genetic heterogeneity). It is likely that some of the genes recently identified in the NOD mouse are minor susceptibility genes of these types.

IV. The Role of the Environment: Does it Trigger or Just Modulate the Anti-β-Cell Autoimmune Response?

A. Introduction

1. Evidence for the role of environmental factors. Several lines of evidence point to a major role of environmental factors in the pathogenesis of IDDM. First, more than 60% of identical twins are discordant for the disease, and it is quite unlikely that this is due to differential somatic rearrangement of T cell receptors. Second, disease frequency varies enormously from country to country (86), and these differences cannot simply be explained by ethnic genetic differences since migrants from countries with a low IDDM frequency to countries with a high frequency are more susceptible than their compatriots (87). Intriguingly, northern countries are more exposed to the disease than southern countries (86); it will be critical to discover the factor(s) responsible for this North/South gradient. Third, a number of apparently nonimmunological interventions can increase or decrease the disease rate in animal models: specific diets [low essential fatty acid (88) or protein intake (89, 90)] and several viral infections (91–95) can reduce disease susceptibility in NOD mice and BB rats, while Kilham's virus (96) and cow's milk (97, 98) can increase it in BB rats. These factors, particularly viral infections, probably explain the variations in disease frequency between NOD colonies (6).

Finally, disease incidence is on the increase in most countries [a 2-fold rise has occurred in Finland over the last 15 yr (99)], strongly pointing to an environmental influence; this holds true even in areas with a distinct genetic background such as Sardinia, where the incidence has recently increased dramatically to values much higher than those in surrounding regions (100).

Not only do environmental factors seem to influence IDDM onset, they can also apparently alter the course of the disease. These factors can be shared by the whole population (climatic factors, hygiene, etc.), or by a given family (*e.g.* eating habits), or be specific to the individual (*e.g.* travels and sexual partners). Retrospective epidemiological studies are difficult to interpret, but prospective testing of candidate environmental factors holds out far more promise. Such a study of cow's milk feeding in the first weeks of life is underway.

2. Trigger or modulator? It is generally agreed that environmental factors are at the rigin of a large number of diseases. This is certainly the case for infectious diseases, even if the genetic background can strongly influence disease expression. The situation is very different in the case of diseases in which nvironmental factors essentially modulate the expression of predisposing genes, either positively (predisposing factors) or negatively (protectiv factors). In the case of triggering factors, disease onset is directly related to the encounter with the environmental factor (usually single and limited in time), which can then be considered as the cause of the disease. In the "modulation" hypothesis, the disease can only appear in the fraction of the population at genetic risk and it is on this population that environmental factors (usually multiple and chronic) exert their positive or negative effect. Available data suggest that IDDM is of the second type.

B. Viruses and IDDM. Interactions with the immune system

A viral origin of IDDM was one of the first etiological hypotheses (101, 102), but the data on which it was based are more complex than initially thought and must now be interpreted in light of data on the autoimmune pathogenesis of the disease. Nonetheless, the viral origin of IDDM remains a central point of debate in the etiology of the disease.

1. Epidemiological data supporting the etiological role of viruses. IDDM onset is often seasonal (103) and could follow outbreaks of certain infections (101, 102). Particular attention has been paid to rubella virus [~ one-third of *in utero* rubella cases develop IDDM (104)] and Coxsackie virus (101, 102, 105, 106). A strain of Coxsackie B virus isolated from a pancreas collected from a single child who died from recent-onset IDDM was able to induce IDDM in mice. It may also be of interest that anti-Coxsackie B virus antibodies have been found in an abnormally high percentage of type 1 diabetics (106).

2. Animal models of virus-induced diabetes. A number of viruses can induce diabetes in various animal species, notably the encephalomyocarditis virus (EMCV), which induces diabetes in several mouse strains (without linkage to the MHC) (107). The effect seems to be mediated by a direct cytolytic effect of the virus, although in the case of some virus variants, diabetes can be prevented by anti-CD4 monoclonal antibodies (108) or irradiation and does not develop in athymic mice (109). This suggests the possibility of an immune phase after the initial direct cytolytic effect of the virus. Other viruses inducing diabetes in animals include reovirus type 1 in mice (with insulitis) (102) and rubella virus in Syrian hamsters (110). Also, it is worth mentioning the endogenous xenotropic retrovirus expression in β-cells of NOD mice (111, 112) and the spectacular triggering of diabetes in the diabetes-resistant DR subline of BB rats after infection by Kilham's virus (96), diabetes apparently caused by a direct cytolytic effect of the virus on β-cells (113).

3. Mechanisms of virus-induced IDDM. Several mechanisms are feasible. The most obvious, clearly demonstrated in several of the models just mentioned (notably EMCV infection in mice and Kilham's virus in DR BB rats), is a direct cytolytic effect of the virus on β-cells. Another mechanism, not exclusive of the first, involves a T cell immune reaction to the virus neoantigens induced at the β-cell surface. This mech-

anism is best illustrated by the model of SV40 transgenic mice expressing the T antigen in β-cells late in ontogeny (31) at a stage of immunological development where exogenous antigens do not induce tolerance. Another possibility is endogenous, vertically transmitted viruses as illustrated by transgenic mice whose β-cells express the LCMV glycoprotein or influenza virus hemagglutinin; these mice become diabetic after viral infection (38, 39) or hybridization with mice transgenic for the antiviral protein TCR (41). It may be worth recalling here that, depending on the virus and (perhaps) the mouse strain, these double transgenic mice require viral infection to become diabetic, suggesting that virus-induced T cell activation may be necessary for diabetes onset, at least in some cases.

Alternative mechanisms are related to molecular mimicry, by which a nontolerized exogenous antigen cross-reacting with a tolerized autoantigen can break down the tolerance to the latter. In the case of IDDM-inducing viruses, virus proteins could conceivably share a sequence with a β-cell autoantigen, as exemplified by the homology between a Coxsackie B viral protein and glutamic acid decarboxylase, a β-cell autoantibody described below (114). Molecular mimicry might also apply to cross-reactivity between an antiviral antibody idiotype and a β-cell autoantigen.

A more trivial interpretation, providing the most likely explanation for the emergence of IDDM after an acute viral infection, is related to the increase in insulin requirements that follows some viral infections: there is no other plausible explanation for the temporal relationship between an acute infection and IDDM onset in most cases, since islet cell autoantibodies are produced several years before the clinical onset of IDDM and consequently long before, not after, the acute infection in question.

4. Viral infections and protection from IDDM in genetically predisposed individuals. Intriguing evidence has recently emerged suggesting that some viruses can protect genetically predisposed animals from diabetes. For example, infection with the mouse lymphocytic choriomeningitis virus (91, 92), the lactodehydrogenase virus (93), or the murine hepatitis virus (94) prevents IDDM in NOD mice when contracted before 2 months of age. These data are in keeping with the observation that both NOD mice (our unpublished data) and BB rats (95) show an increased incidence of the disease when raised in germ-free conditions. The mechanisms of this virus-associated protection are not clear but could involve antigenic competition in the larger sense of the term. For example, viruses could activate the production of immunosuppressive cytokines (of the TH2 type described below). It is important to determine whether the North-South gradient of diabetes incidence mentioned above is partly due to common viral infections; for example, due to the lower temperature and better hygiene, inhabitants of northern countries may be less exposed to infections than those in southern countries, as is the case for hepatitis A virus and cyt megalovirus. This hypothesis is supported by the similar North/South gradient observed for multiple sclerosis, another T cell-mediated aut immune disease, and the inverse South/North gradient observed for carriage of antibodies to hepatitis A virus used as a marker of infection by water-borne pathogen (95a).

5. Conclusions. It is difficult to unify so diverse and sometimes contradictory data and hypotheses. It can, however, be assumed that some viruses nonspecifically protect against diabetes, while others can induce the disease, either by a direct cytolytic effect or through the T cell response to viral neoantigens expressed at the β-cell surface. In spite of convincing experimental models, however, there is no convincing evidence for a direct pathogenetic role of a virus in human IDDM, at least in the vast majority of cases in which the involvement of the immune system is well documented (see below). In contrast, a chronic viral infection of β-cells is possible, where β-cell neoantigens stimulate a T cell response like that observed in the SV40 transgenic model described above (31). A vertically transmitted virus could also be involved since, as illustrated by the LCMV or influenza virus hemagglutinin transgenic models (38, 39, 41), fetal xpression of viral neoantigens by β-cells does not necessarily induce tolerance to the viral antigens. This observation indicates that the immune response to the neoantigen(s) cross-reacts with β-cell autoantigens in uninfected individuals, since diabetes can be transferred to nondiabetic individuals presumably not infected by the virus. Insulitis reappears rapidly in syngeneic pancreas transplants derived from a monozygotic twin placed in a diabetic patient (115). Similarly, IDDM has been described after allogeneic bone marrow transplantation from a diabetic donor (116, 117). These observations are in keeping with those made in NOD mice and BB rats showing recurrence of IDDM when normal allogeneic islets are grafted in conditions avoiding allograft rejection (118, 119). Note, however, that one cannot rule out in all these settings the possible viral contamination of the graft, which casts a doubt on the interpretation of these results.

C. Mycobacteria and IDDM

Freund's complete adjuvant (CFA), which consists of mycobacteria incorporated in a water-in-oil emulsion, completely prevented the onset of IDDM when injected in young NOD mice (120, 121) and BB rats (122). Spleen cells from CFA-protected animals suppress responses of cocultured syngeneic control spleen cells to mitogens *in vitro* (120, 121) and protection can be transferred by spleen cells from th CFA-treated animals to naive animals (123). The nature of the protective cells is still uncertain (macrophages, NK cells, TH2 cells). These data, which have been reproduced with Bacillus-Calmette-Guerin (BCG) vaccine in NOD mice (124), were sufficiently convincing to warrant a therapeutic trial in human prediabetes with BCG (124a).

D. Toxic agents

As mentioned above, several toxic agents show β-cell selectivity and induce IDDM at doses not provoking significant extrapancreatic toxicity (125). This is the case of STZ (18) and alloxan (19). Another agent, Vacor (a rodenticide), has also been shown to induce IDDM at the high doses us d in suicide attempts (126). Pentamidine, a drug given to AIDS

patients for prophylaxis of *Pneumocystis carinii* pneumonia, may have a similar effect (127). However, there is little evidence that any toxic agent, whatever its mechanism of action, is at the origin of common forms of IDDM. At most, some toxic agents could act by amplifying the anti-β-cell autoimmune response, as in the low-dose STZ model described above (20), since diabetes onset is accelerated in NOD mice at STZ doses lower than those inducing diabetes in conventional strains (25, 26).

E. Food constituents. The cows' milk hypothesis

Diets are known to influence glucose metabolism, with obvious consequences for diabetics. A number of diets, independent of their direct glycemic effects, have recently been shown to delay the onset of IDDM in NOD mice and BB rats, probably by interfering with the anti-islet immune response. This is the case for low essential fatty acid (89) and protein diets (90).

Conversely, cow's milk accelerates the course of diabetes in BB rats, while lactalbumin-free diets are protective when administered for the first 2 to 3 months of life (97, 98). A role for the whey protein BSA has been suggested, because early induction of tolerance to BSA prevents IDDM and anti-BSA immunization accelerates it in BB rats (see Ref. 128). Much attention has been paid to the possibility that a BSA-related protein could represent an important triggering factor for human IDDM. Anti-BSA antibodies are found in diabetics more frequently than normal (using a particle concentration fluoroimmunoassay) (128, 129). Diabetic children have an abnormally high frequency of immunoglobulin A (IgA) antibodies to β-lactoglobulin (130–132). It is important to mention, however, that these findings are based on a precise methodology and have not always proven easy to repeat (133). Anti-BSA antibodies in diabetics recognize a peptide sequence (ABBOS) containing 17 amino acids in a region of the BSA molecule extending from position 152 to position 168, *i.e.* the site of the major sequence difference with human, mouse, and rat albumin. This peptide sequence cross-reacts with a 69 kilodalton (kDa) β-cell autoantigen (p69), which has recently been cloned independently by two laboratories using anti-BSA (141a) or anti-islet cell antibody-positive diabetics' sera (134) to screen a human pancreas cDNA library. This cross-reaction could explain the stimulation of the anti-islet T cell response by cow's milk in the first week of life (molecular mimicry). It should be noted, however, that at variance with this hypothesis, recent onset diabetics do not show T cell hypersensitivity to BSA or ABBOS (133). Nonrandomized data indicate that exclusive breast-feeding, with delayed exposure to infant formula based on cow's milk, significantly reduces the risk of diabetes in Finnish children (129). A prospective randomized trial has been set up to confirm these data.

F. Stress

There is mounting vidence that psychoaffective events can influence immunity, and some groups have focussed on stress as a possible trigger of IDDM (135–138). It has thus recently been shown that acute stress can accelerate the onset f diabetes in NOD mice (137), whereas raised environmental temperature reduced it (138).

G. Sex hormones

Diabetes is much more common in female than in male NOD mice (5) and its onset is accelerated in males by castration, particularly when combined with thymectomy (139). Conversely, androgen treatment of female mice prevents diabetes (140). The mechanism of action of sex hormones on the immune system is unclear but could involve an effect on immunoregulatory networks: male NOD mice develop insulitis, but most do not become diabetic unless given cyclophosphamide, a drug known to affect suppressor cells (11, 12).

V. Does IDDM Fulfill the Criteria of an Autoimmune Disease?

A. Definition of autoimmune diseases

Autoimmune diseases are diseases due to the pathogenic effect of autoantibodies or autoreactive T cells that provoke inflammation, functional alterations, or anatomical lesions. They must be distinguished from diseases associated with autoimmune manifestations not directly related to disease pathogenesis.

B. Criteria defining autoimmune diseases

Four criteria usually have to be met to consider a disease as autoimmune (141).

1. The disease state can be transferred by the patients' antibodies or T cells.
2. The disease course can be slowed or prevented by immunosuppressive therapy.
3. The disease is associated with manifestations of humoral or cell-mediated autoimmunity directed against the target organ.
4. The disease can be experimentally induced by sensitization against an autoantigen present in the target organ, which presupposes the knowledge of the target autoantigen.

Points 1 and 2 are mandatory. Points 3 and 4 are important but less critical. In fact, only a few so-called autoimmune diseases fulfill all four criteria (one example is myasthenia gravis due to anti-acetylcholine receptor autoantibodies).

C. IDDM as an autoimmune disease

Human IDDM fulfills three of these criteria and indirect arguments exist in animal models for the fourth.

1. Diabetes transfer. Diabetes can be transferred in NOD mice and BB rats into nondiabetic syngeneic animals by spleen cells from diabetic animals (9, 10, 142–144). More precisely, it has been shown using purified T cell preparations and T cell clones derived from spleen or islets of NOD mice that the transfer was exclusively due to T cells (142, 144–146).

We shall see below the phenotype and repertoire of such diabetogenic T cells. Similarly, appearance of diabetes has been observed in man after pancreas transplantation between identical twins (115). Such diabetes is likely due to infiltration of the transplanted pancreas by the recipient autoimmune cells (whether or not they have been reactivated by reexposure to pancreas autoantigen). One should also mention diabetes observed after allogeneic bone marrow transplantation with a diabetic donor (116, 117). The situation is less pure in the latter models since one cannot exclude that non lymphoid cells present in the donor bone marrow could be responsible for the transfer.

2. *Effect of immunosuppression.* Insulin β-cell damage can be slowed by immunosuppressive therapy, notably cyclosporine (147, 148) and many other immunosuppressive agents essentially active at the T cell level in NOD mice, BB rats (149, Tables 3 and 4), and man (Table 5). The effect is better observed when the treatment is applied early, which is obviously much more difficult to achieve in man than in animal models, but some significant effect is still seen at the disease onset (Table 3).

3. *Manifestations of anti-β-cell autoreactivity.* There is evidence for both islet-reactive autoantibodies and T cells [*e.g.* islet cell antibodies (ICA) (150), glutamic acid decarboxylase (GAD)-reactive antibodies (151), and T cells (152, 153)].

a. Autoantibodies. Diabetic patients and rodents mount a multifaceted humoral immune response to islet cells. Autoantibodies are found against a wide array of membrane and cytoplasm constituents of β-cells, including insulin (anti-insulin autoantibodies are detected before starting insulin therapy) (154), proinsulin (155), and GAD (151). The most commonly screened antibodies, whose description in 1974 (150) led to the first strong evidence for the autoimmune origin of IDDM, are the so-called ICAs detected by indirect immunofluorescence on human pancreas sections. ICAs bind to the cytoplasm of β-cells [perhaps to gangliosides (156)], but they also usually bind to the cytoplasm of other islet endocrine cells. There are, however, "restricted ICAs" that selectively bind to β-cells (157), which essentially include antibodies directed at GAD (see below). Some interest was initially paid to antibodies directed against islet surface antigens that can be cytotoxic to β-cells (158) or inhibit insulin release by β-cells in the presence of complement (159), but these antibodies are poorly characterized.

b. T cells. Paradoxically, although T cells apparently play the central role in IDDM pathogenesis, few data have been published on T cell reactivity to islet antigens in humans. Of note are pioneering studies using the leukocyte migration assay with islet extracts (160) and, more recently, proliferation assays using human islets, fetal pig islets (161, 162), GAD (152, 153), and hsp 65 (our unpublished observations). The anti-islet T cell response has been best documented in the NOD mouse and the BB rat, where transfer of diabetes can be obtained with purified T cell populations (142, 143, 145), culminating in the production of pathogenic islet-specific T cell clones (144, 146). Successful transfer requires the simultaneous presence of CD4 and CD8 cells when using irradiated recipients that are the most immunoincompetent (142, 163, 164).

4. *Immunization and tolerance.* Criterion 4 of autoimmune diseases (reproduction of the disease by sensitization against an autoantigen) cannot be met in human diabetes and has very partially been met in animal models, probably due to the uncertain knowledge of the target autoantigen. This is not an absolute criterion even if such a demonstration would greatly aid our understanding of IDDM pathogenesis. The induction in normal animals of insulitis by anti-insulin sensitization (165) and of transient diabetes by immunization against a hsp 65-derived peptide (166) opens the way in this direction. Additionally, two recent studies have shown that insulitis and diabetes can be prevented in NOD mice by injecting them with soluble recombinant GAD at 3 weeks of age either intravenously (167) or intrathymically (168).

5. *Indirect evidence.* The following indirect evidence exists to support the autoimmune nature of human IDDM: 1) infiltration of the islets of Langerhans by mononuclear cells (insulitis) (169–171); 2) common association of IDDM with other "classical" autoimmune diseases, notably thyroiditis (47); 3) association of IDDM with HLA genes (64–71), which are known to be associated with most autoimmune diseases; and 4) anomalies of the immune system not directly linked to islet cell autoreactivity in human diabetics, such as augmented levels of activated T cells (DR+ and IL-2R+) (172, 173), circulating IL-2 receptor (173, 174), and CD5+ B cells (175). Other abnormalities have been described in animal models, such as lymphocytopenia (14) and increased NK cell activity (176) in BB rats, thymic anomalies in NOD mice (177–181) and BB rats (182), and decreased IL-4 production (183) and delayed T cell apoptosis in NOD mice (80).

VI. β-Cell Target Autoantigens

A. Introduction: the role of β-cell autoantigen(s) in sensitization and lesion formation

The identification of target autoantigens in IDDM is a major challenge for pathogenesis, immunological diagnosis, and immunotherapy. Several candidate autoantigens have been described, but none has so far convincingly been shown to be 'the diabetes autoantigen.' The existence of a precise target autoantigen epitope is suggested by the IDDM association with specific HLA alleles (MHC immune response genes are specific for a given epitope) but one might argue that HLA disease control is not necessarily antigen-specific (MHC genes other than class II genes may explain the HLA-IDDM association). Our recent demonstration that alloxan-treated NOD mice, which lack β-cells, can no longer sustain the survival of pathogenic T cells (184) also supports the hypothesis that the autoimmune response in IDDM is driven by a β-cell autoantigen, as is presumably the case in many if not all organ-specific autoimmune diseases (185). Neonatal thyroidectomy prevents the spontaneous production of antithyroglobulin autoantibodies normally synthesized in the obese chicken (186).

TABLE 3. Immunotherapy of diabetes in NOD mice

Agent	References	Prevention (treatment started ≤3 months of age)	Prevention of diabetes transfer (treatment of the recipient)	Prevention of cytoxan-induced IDDM	Treatment of overt diabetes (treatment started after the onset of hyperglycemia)
Immunomanipulation					
Neonatal thymectomy	303	+			
Allogeneic bone marrow transplantation	226	+			
Backcross to nude mice	9	+			
Backcross to scid mice	10	+			
MHC transgenes	53–58	+			
Intrathymic islet grafting	296	+			
CD4 T cells	246		+		
Immunosuppressive agents					
Cyclosporin	304	+			±/−
FK506	305, 306	+		+	
Deoxyspergualin	307			+	
Rapamycin	308	+			−
ALS	278				+
Monoclonal antibodies					
αCD3	294	+			+
αTCR	262	+		+	+
αVβ8	220			+	
αCD4	118, 293, 309–312	+	+		+
αCD8	313 + (our unpublished data)	+	+	+	+
αclass I	314			+	
αclass II	250	+	+ (neonate)	+	
αIL-2R	315	+			
αCD45RA	178	+			
αγIFN	237, 238		+	+	
α-IL-6	238				
Cytokines					
IL-1	268	+	+		
IL-4	183	+			
TNFα	269, 270	+	+		
IL-2 toxin	316		+		
Miscellaneous					
CFA	120–121, 124a	+			
BCG	124	+		+	
Antioxidants	317	+ (with steroids)			
Aminoguanidine (NO inhibition)	271		+		
Vitamin D_3	318	+ (insulitis)			
Gangliosides	319	+			
Con A	320	+			
hsp65/peptide	16, 17	+			
Insulin (parenteral)	201, 202	+			
Insulin (oral)	200	+			
Diets	88–90	+			± −
Nicotinamide	321	+			
Immunoglobulins	322	+			
Silica	323	+		+	
Peptides	295	+			
Viruses	91–94	+			

+, Suppression of diabetes; −, no effect.

TABLE 4. Immunotherapy of diabetes in BB rats

Agent	References
Immunomanipulation	
Neonatal thymectomy	324
Allogeneic bone marrow transplantation	225
Intrathymic islet grafting	228, 229
Lymphocyte transfusion	254
Immunosuppressive agents	
Cyclosporin	325–327
Anti-lymphocyte sera	328
Anti-class II monoclonal antibodies	329
Anti-IFNγ monoclonal antibodies	239
Miscellaneous	
Total lymphoid irradiation	330
TNFα	331
Low essential fatty acid diet	88
Low protein diet	89, 90
Insulin (parenteral)	203–205

TABLE 5. Immunotherapeutic trials in human IDDM

		Reference
Immunosuppressive agents		
Cyclosporin		147, 148, 297, 300, 301
combination	+ nicotinamide	332
	+ bromocriptine	333
FK506		334
Steroids		335
Azathioprine		299
combination	+ corticoids	298
	+ thymostimulin	336
OKT3		149
IL-2 toxin		149
Miscellaneous		
Nicotinamide		290, 291, 292
Subcutaneous insulin		206
Intravenous immunoglobulins		337
Lymphocyte transfusion		338
Pancreatic irradiation		339
Thymopoietin		340

Alternatively, the anti-islet response could be part of a more global immune hyperreactivity, as in the rat model of generalized autoimmunity obtained after thymectomy and irradiation (23, 24). In this model, pathogenic anti-islet autoimmunity is only the expression of exaggerated physiological autoreactivity due to the loss of immune regulatory function, with no apparent requirement for an autoantigenic driving force.

An intermediate possibility is that β-cell autoantigens do indeed drive the anti-β-cell autoimmune response but that several autoantigens (each with a limited number of dominant epitopes) intervene concomitantly. For unknown reasons (*e.g.* a viral infection), the β-cells might become abnormally immunogenic and stimulate a strong autoimmune response to several of its molecular constituents, provided there is the relevant MHC molecule to present them to T cells. In this hypothesis, either one of these triggering β-cell autoantigens plays a dominant role or MHC genes are not involved in disease susceptibility through conventional Ir genes. There is little room for multiple unrelated autoantigens to share the same precise HLA binding epitopes.

B. Primary and secondary autoimmunization. B and T cell epitopes

It is unlikely that the whole B and T cell response toward a large number of β-cell autoantigens observed in diabetics is primary (or pathogenic). The initial T cell-mediated β-cell lesions probably induce the release of degradation products that in turn elicit the production of secondary B or T cell immune responses. This is suggested by the chronological appearance of T cell proliferative responses to several β-cell autoantigens in the NOD mouse (168). Tolerance induction to the first of these autoantigens prevents onset of reactivity to other autoantigens without reciprocity. This is also probably the case for the anti-islet autoantibodies discussed above. The problem is further complicated in the case of T cells by the fact that these secondary immune responses could contribute to the development of the β-cell lesion and play a significant role in the chronicity of disease. It should be mentioned at this stage that, for obvious reasons of feasibility, most studies aimed at the identification of IDDM autoantigens involve the use of autoantibodies for screening, whereas the initial triggering autoantigen(s) and target autoantigen(s) are recognized by T cells. This is a major pitfall since T and B cell epitopes differ radically: T cell epitopes are sequential whereas B cell epitopes are conformational (187). In addition, T cells can recognize intracytoplasmic proteins that are processed and then exposed at the cell surface in conjunction with MHC molecules, whereas antibodies can only be pathogenic *in vivo* after binding to cell surface molecules.

C. Candidate autoantigens

A number of putative β-cell autoantigens have recently been characterized.

GAD is an enzyme controlling the biosynthesis of the inhibitory neurotransmitter γ-aminobutyric acid. It has recently been identified (151) as one of the 64 kilodalton (kDa) antigens previously detected by immunoprecipitation of islet extracts by diabetics' sera (188). GAD exists in two isoforms of 65 and 67 kDa (189). It is present in β-cells and the brain, and its sequence shows major homology both between the two isoforms and between mammalian species (189, 190). Anti-GAD antibodies were initially found (at high titers) in the stiff-man syndrome, a neurological disease often associated with ICAs and sometimes IDDM (151). They are also found at lower titers (using various techniques: enzyme trapping, immunoenzymatic assays, etc.) in 60–70% of diabetics (191–193) and in most ICA+ prediabetics (194, 195). As mentioned above, T cell proliferation is induced *in vitro* by recombinant GAD preparations in IDDM patients (152, 153), but the antigen specificity of the proliferation remains to be proven with highly purified material. One must formally exclude contamination by highly mitogenic end toxin of the bacterial recombinant preparation used in these stud-

ies. Although there is little doubt that GAD is one of the major β-cell antigens, the role of this antigen in the pathogenesis of human IDDM remains to be proven. Indeed, anti-GAD antibodies do not appear to be more predictive than ICAs of diabetes onset in prediabetics (194, 195), and a protective role among ICA+ subjects has been indicated by recent studies (196). Conversely, a pathogenic role could be given to GAD-reactive T cells. Recent data obtained in the NOD mouse indicate that administration of GAD in 3-week-old mice, either intravenously (167) or intrathymically (168), prevents the onset of insulitis and diabetes.

A 37-kDa protein is immunoprecipitated by diabetic patients' sera together with a 50-kDa protein instead of the 64-kDa band when islet extracts are treated with trypsin (197). Antibodies directed against the 50-kDa species recognize GAD. They are absorbed by recombinant GAD65, and their presence strictly correlates with that of anti-GAD antibodies. Conversely, antibodies directed against the 37-kDa protein are apparently distinct from anti-GAD antibodies. They are not absorbed by recombinant GAD, suggesting that the 37-kDa protein is derived from a 64-kDa molecule distinct from GAD (198). The anti-37-kDa antibodies seem to be better predictors of diabetes in prediabetics than anti-GAD antibodies (196).

Insulin is a logical candidate for an IDDM autoantigen since it is the best established β-cell-specific differentiation antigen. Its role in the pathogenesis of the disease appears initially unlikely though, since insulin is essentially expressed in the β-cell cytoplasm. However, we have seen that such cytoplasmic antigens can be processed and recognized by T cells. Anti-insulin autoantibodies are often found in prediabetics before treatment with insulin (154). Immunization of normal animals of different species induces insulitis (165), and sensitization of prediabetic NOD mice against insulin can protect them from diabetes when either the parenteral (199) or the oral (200) route is used. This protective effect is presumably linked directly to the immunogenicity of insulin at least when used parenterally, since the functionally inactive insulin B chain can be used instead of native insulin in parenteral sensitization experiments (199). This effect of insulin should be distinguished from the above-mentioned protection conferred by subcutaneous injections of insulin that probably act directly at the β-cell level (201–206).

hsp 65 (65-kDa heat shock protein) (16) and one of its constitutional peptides (17) have been reported to accelerate the onset of diabetes in NOD mice and even to induce *de novo* diabetes in C57BL/6 mice when coupled to a carrier protein (166). The diabetes thus induced is, however, transient and NOD mice are ultimately protected from diabetes. Disease acceleration and protection can be transferred by hsp 65-reactive T cell clones (17), suggesting that the protection could relate to a mechanism of T cell vaccination, in which mice become sensitized against the T cell receptor of hsp 65-reactive T cell clones. hsp 65 Has been found in β-cells and c uld be a new target autoantigen (T cell epitope). Alternatively, it could act via molecular mimicry.

p69 Protein has been mentioned as a β-cell autoantigen cross-reacting with BSA (128, 129, 134). The anti-p69 response could b stimulated by c w's milk protein administered during the first weeks of life, again via molecular mimicry.

A 38-kDa protein isolated from β-cell insulin secretory granules has been shown to stimulate T cell proliferation in human diabetics' lymphocytes, giving rise to the production of T cell clones (207, 208). This protein could contain important T cell epitopes.

Other candidate antigens include peripherin, a neurone cytoskleton molecule (168, 209), carboxypeptidase H (168, 210), and the ICA-reactive gangliosides (156).

VII. The Loss of Self-Tolerance to β-Cell Antigens

A. Tolerance to self

It is a major feature of the immune system that B and T cells are physiologically tolerant to most self-antigens (*i.e.* there is no pathogenic autoimmune response). This state of T cell self-tolerance is mainly controlled in the thymus, where self-reactive T cell clones that expanded after contact with self MHC molecules present on the thymic epithelium and stroma (positive selection) are eliminated by autoantigen-driven apoptosis (negative selection) (211, 212). This phenomenon does not, however, eliminate all autoreactive clones, particularly those reacting toward subdominant or cryptic epitopes (213) and autoantigens not present in sufficient concentrations in the thymus. These autoreactive clones are controlled by either a phenomenon known as T cell anergy (the autoreactive cells are present but are not activated after binding the antigen) or by the effect of suppressor mechanisms (211–213). There are several examples in transgenic mice where T cells reactive with antigens expressed on β-cells are reactive with the antigens *in vitro* but not *in vivo*. These cells are not truly "anergic" but may be "ignorant" and hence do not engage in an immune response if they are not properly activated (see above the LCMV transgenic mouse model). Finally, the breakdown of self tolerance that characterizes autoimmune diseases can thus occur through three major mechanisms: insufficient intrathymic negative selection, bypass of peripheral anergy, or defective suppression (211–213).

B. T cell repertoire

Most information on islet-reactive T cells in IDDM is derived from the study of NOD mice. This has been facilitated by the production of a number of islet-specific T cell clones, mostly of the CD4 phenotype (144, 146, 214). Some of these clones have been shown to be diabetogenic after transfer into irradiated adult (146) or nonirradiated y ung NOD recipients (144). The TCR of one of these CD4 clones was recently used as a transgene (215); transgenic mice showed rampant insulitis at a faster rate than the transgene negative NOD littermates but only borderline and inconsistent hyperglycemia.

Encephalitogenic T cell clones obtained after immunization with myelin basic protein use restricted Vα and Vβ TCR

genes (216). It was thus important to search for a possible restriction of Vα and Vβ gene usage of TCR of T cells involved in IDDM pathogenesis. Several approaches have been taken. Phenotypic studies using indirect immunofluorescence with selected anti-Vβ monoclonal antibodies (217) or dot blot hybridization (218) on pancreas sections or extracts are difficult to perform and have yielded no evidence f restriction. The T cell clones mentioned above do not show any clear preference for a given Vα or Vβ (214, 219). The only two studies that have revealed some restriction were based on diabetes prevention by anti-Vβ monoclonal antibodies. An anti-Vβ8 monoclonal was reported to prevent cyclophosphamide-induced diabetes (220) [an observation not reproduced by another team (221)] and an anti-Vβ6 monoclonal inhibited diabetes transfer in irradiated mice (222). It is interesting to note, in this context, that NOD mice backcrossed to other strains to give a strain that congenitally lacks approximately one-half of the conventional TCR Vβ alleles (including Vβ8 but not Vβ6) still develop diabetes (223). Finally, we shall have to await results of studies in progress with anchored polymerase chain reaction at early stages of insulitis to know whether the TCR of T cells infiltrating NOD islets show restricted usage of any particular TCR fragment, at least in the initial stages. This is an important question from both the fundamental and therapeutic viewpoints, since if a restriction exists, one could envisage preventing IDDM by targeting the minor T cell subset expressing the Vβ gene in question. The T cell oligoclonality can also be studied by analyzing TCR junctional sequence variability. Results obtained in our laboratory (manuscript in preparation) indicate that such oligoclonality might exist initially at the islet level but polyclonality rapidly spreads over the pancreas.

C. Location of the anomaly(ies) leading to the pathogenic anti-β-cell autoimmune response

There is no indication in IDDM, as in other organ-specific autoimmune diseases, that the target autoantigen is abnormal. In fact, transplantation studies mentioned above (115–119) showing that destructive insulitis can be transferred to n n-diabetes-prone mouse, rat, or human pancreas indicate that the anomaly is located in the immune system. This is corroborated by the observation that reconstitution of (BALB/c × BG)F1 normal mice with stem cells and thymus from NOD mice results in autoimmune insulitis of the (normal) host pancreas (224). Similarly, reciprocal allogeneic bone marrow transplantation between BB rats and a non-autoimmune rat strain shows that the defect leading to diabetes lies in the bone marrow stem cells (225).

All experimental data converge to suggest that the defect is most strongly expressed at the T cell level. The disease is prevented in NOD mice and BB rats by neonatal thymectomy, backcross to athymic animals, and administration of various anti-T cell antibodies (Table 3). Diabetes can be transferred to healthy recipients by purified T cell populations (142, 143, 145) or T cell clones (144, 146). The question then arises as to whether the anomaly is located 1) at the T cell precursor level (in the bone marrow), 2) in the thymus (unable to perform normal negative selection or to differentiate effector or regulatory cells), r 3) at the level of the MHC-autoantigen interaction, which would generate molecular complexes that are highly immunogenic for T cells of diabetes-prone individuals. Evidence has been found in favor of all three hypotheses.

Bone marrow precursor cells contain the "germ" of diabetogenicity, since transplantation of NOD mouse or BB rat bone marrow to nondiabetic strains (after irradiation) leads to diabetes (224, 225), and bone marrow transplantation from human diabetics may lead to rapid diabetes onset in the recipient (116, 117). Conversely, transplantation of 'normal' allogeneic bone marrow prevents diabetes in NOD mice and BB rats (225, 226).

This does not rule out an intrinsic thymus defect, several of which have been identified in the NOD mouse: 1) d ficient *in vitro* thymocyte proliferation in response to antigens and mitogens shown recently to be linked to deficient regulation of the $p21^{ras}$ activation pathway (177); and 2), abnormal proportions of CD45RA+ T cells among mature thymocytes (178). These thymocyte abnormalities could relate to the bone marrow defects just discussed. This is less likely the case for abnormal extracellular matrix (with large perivascular spaces filled with lymphocytes) (179, 180), and reticulum (181) and deficient thymic hormone secretion (179). All these anomalies could indicate a defective thymic microenvironment. In the same vein is the decreased expression of class II MHC molecules observed in some areas of the BB rat thymus (182).

The role of MHC molecules has already been discussed. Their central contribution to diabetes susceptibility is clearly established, but it is certainly not sufficient in itself, since the majority of subjects with a predisposing HLA allele never develop the disease. Additionally, one should not equate the HLA-IDDM association to the presence of predisposition immune response genes (HLA-autoantigen peptide presentation), since MHC genes can be involved at several levels not directly related to peptide recognition by T cells.

D. Defective negative selection

This hypothesis is illustrated by the SV40 transgene mouse model mentioned above (31), in which late expression of the T SV40 antigen (after intrathymic negative selection has taken place) leads to anti-T antigen sensitization and insulitis, inasmuch as the T antigen is selectively expressed on β-cells. This mechanism could apply to virus-induced neoantigens. There is little evidence, however, for the existence of such neoantigens either in NOD mice and BB rats or in human IDDM. There is apparently no major abnormality of distribution of T cells expressing the various Vβ fragment expression in NOD mice (218) or BB rats (227), as could have been anticipated if negative selection by a superantigen had created major gaps in the T cell repertoire. In fact, islet-reactive T cells having escaped negative selection are present in normal individuals, as demonstrated by the onset of diabetes in n n-autoimmune-prone rats after thymectomy and irradiation (28, 29) and by the induction of diabetes in normal mouse strains after sensitizati n to hsp 65 peptides (166). In

conclusion, although one cann t exclude it formally, there is little evidence so far in IDDM of a failure for negative selection of β-cell reactive clones. One can assume that physiologically β-cell target autoantigens are not present in the thymus at sufficient concentrations to induce negative selection of the responding T cell clones or that these antigens may be present in the thymus but diabetogenic epitopes are subdominant or cryptic and do not give rise to negative selection. The possibility demonstrated in both the NOD mouse and the BB rat to prevent the onset of IDDM by placing islet grafts (228, 229) or soluble GAD (168) intrathymically is compatible with such a hypothesis.

E. Breakdown of T cell anergy

Anergized T cells are not activated by antigens presented in normal conditions but can differentiate in the presence of large amounts of IL-2. There is no direct evidence of such a mechanism in IDDM, except for the unconfirmed acceleratı n of diabetes in BB rats after IL-2 administration (230). However, hyperexpression of class I MHC molecules (170, 231–233) and, more controversially, aberrant expression of class II molecules (170, 231, 233–235) could conceivably favor more efficient presentation of β-cell antigens to T cells and thus break down the physiological anergy of islet-reactive T cells (if indeed MHC class II-expressing β-cells can present antigens). The role of IFNγ in MHC class II expression is suggested by the *in vitro* induction of HLA class II molecules in human islet cells by IFNγ (plus TNF) (236) and by the prevention of diabetes by administration of anti-IFNγ monoclonal antibody in NOD mice (237, 238) and in BB rats (239). One must, however, interpret these data with care, even if aberrant expression of class II MHC molecules in β-cells can indeed provoke autoimmune (transferable) insulitis, as shown by the IFNγ transgenic model (32, 33). Alternatively, aberrant expression of class II MHC molecules could be a secondary phenomenon: activated T cells present in the islets produce IFNγ (240) that could induce the aberrant MHC class II molecule expression. Our observation (241) that class II molecule expression appears within a few days after adoptive transfer of diabetogenic spleen cells on pancreatic endothelial cells illustrates this possibility. The absence of abnormal expression of class II MHC molecules reported in prediabetic NOD mice (217, 235) and BB rats (233) and the absence of 'autoimmune' type diabetes in transgenic mice expressing class I (42) or class II (43, 45) MHC molecules in β-cells is compatible with such an alternative hypothesis but in no way proves it. The MHC molecule expression could be too weak in the rodent spontaneous models to be detected by the immunofluorescence technique used in the experiments mentioned (other results reported in Ref. 234) and too high in the transgenic mouse models to provide meaningful information. It should also be mentioned at this stage that T cell-mediated destruction of β-cells can be obtained in the absence of CD4 T cells and MHC class II molecules. Mice that were class II-deficient after a targeted disruption of the $A\beta^b$ gene were bred to transgenic mice expressing the LCMV glycoprotein in β-cells. Such transgenic class II-deficient mice developed diabetes after infection with LCMV (242).

The significance of class I molecule hyperexpression (more consistent in the experimental setting) is complicated by our failure to understand the way in which class I-restricted CD8+ cells contribute to the pathogenesis of IDDM (see below).

Another interesting mechanism is based on the phenomenon of molecular mimicry already mentioned for GAD [cross-reactivity with a Coxsackie B viral protein (114)] and p69 (cross-reactivity with BSA) (128, 129). In this mechanism, the extrinsic antigen to which T cells are not tolerant serves as a carrier for the tolerized cross-reactive T cell epitopes of the autoantigen, leading to a bypass of self-tolerance. It will be important to characterize further th cross-reactive epitopes. Some data have been reported for BSA and p69: the ABBOS peptide is a 17-amino acid residue long peptide shared between BSA and the β-cell p 69 antigen (129). The case of GAD is less well documented since th sequence homology is modest (114). If this molecular mimicry holds true, it would remain to be learned how the chronic autoimmune T cell response is maintained after the disappearance of the cross-reactive external antigen. In the well documented case of rheumatic fever the autoimmune reaction ceases when the antigen (streptococcus) disappears. Perhaps one could think that the initial anti-β cell immune response triggers an anti-idiotype response that would perpetuate the anti-β-cell response within idiotype networks the initial response or more simply that the initial lesion inducing spread sensitization against other β-cell autoantigens released by the first aggression.

F. Defective suppression

The existence and function of suppressor T cells have been the subject of a heated debate among immunologists over the last 10 yr. A number of experimental data suggest that a defect of these suppressor cells might contribute to the onset of diabetes in rodent models of diabetes (243).

In the NOD mouse, diabetes onset is accelerated by thymectomy performed at 3 weeks of age (244) and by administration of cyclophosphamide (11, 12), a drug known f r its selective effects on suppressor T cells. Diabetes transfer is only obtained in immunodeficient recipients, *i.e.* neonates (142) and adults that have been sublethally irradiated (144) or thymectomized and treated with an anti-CD4 monoclonal antibody (245). One can prevent diabetes transfer by spleen cells from diabetic mice by preinfusion of CD4 spleen cells from nondiabetic syngeneic mice (246). CD4 and CD8 suppressor clones have been reported (247–249), as has the production of a suppressor factor (249). Treatment of young NOD mice with an anti-class II monoclonal antibody protects them from diabetes, and this protection is transferable to non-antibody-treated mice by infusion of CD4 T cells from protected mice (250). Similarly, staphylococcal superantigens (SEA and SEB) prevent th onset of diabetes in NOD mice (251), and this protection is also conferable to naive NOD mice by transfer of CD4 T cells from superantigen-treated mice. Also of interest here is the intriguing observation that

diabetes can be prevented in NOD mice by injection of autologous spleen cells exposed *in vitro* to cyclosporin and IL-2 (252).

In the BB rat the disease is accelerated by the administration of an anti-RT6 monoclonal antibody (253) and prevented by transfusion of lymphoid cells from diabetes-resistant DR BB rats (254).

The mechanisms of this defective suppression are still unknown but could involve an abnormal shift of TH2 cells toward TH1 cells of the islet-reactive CD4 T cells. It has been shown that CD4 T cells comprise two subsets—TH1 cells (that produce IL-2 and IFNγ) and TH2 cells (that produce IL-4 and IL-10)—that oppose each other by reciprocal down-regulation. TH1 cells are essentially involved in cell-mediated immune responses, whereas TH-2 cells are involved in helping antibody-forming cells (255). The abnormal shift from TH2 to TH1 islet-reactive T cells is supported by the low IFNγ/IL-4 ratio found in noninvasive insulitis, contrasting with a high ratio in invasive insulitis (240), and by the recent observation that IL-4 (whose production is deficient in the NOD mouse thymus) reverses the T cell proliferative unresponsiveness in NOD thymocytes and delays the onset of diabetes in NOD mice (183). This hypothesis is also in keeping with the inverse relationship between humoral and cellular immunity to GAD in subjects at risk for IDDM (194).

The following findings also support the role of suppressor mechanisms: I-A^k transgenic mice that are protected from diabetes (53) become diabetic after cyclophosphamide treatment, and their spleen cells can transfer diabetes in immunodeficient hosts (256); similarly, spleen cells from I-A^d transgenic NOD mice that are protected from diabetes prevent the diabetogenic capacity of splenocytes from overtly diabetic NOD mice (257). Introduction of I-E in transgenic NOD mice also protects from diabetes (56, 57) through a mechanism that could involve suppressor cells.

G. Conclusions

It is difficult to formulate a global hypothesis explaining the loss of self-tolerance to islet antigens in diabetic subjects. The disease is heterogeneous and multifactorial: several mechanisms may simultaneously be at work, superimposed on a particularly efficacious MHC-controlled recognition of β-cell autoantigen peptides. An attractive hypothesis is a particularly immunogenic expression at the β-cell surface of a subdominant or cryptic autoantigen not having induced intrathymic negative selection. This abnormal expression could be secondary to a viral infection known to modify HLA gene expression through IFN production, but many other cellular events could play a similar role, including endogenous β-cell genetically controlled peculiarities. In this case, as mentioned above, it might be that more than one antigen molecule or epitope shows increased immunogenicity, providing an explanation for the diversified anti-β-cell B and T cell immune response. Alternatively, one epitope could initiate the autoimmune responses [*e.g.* GAD as suggested by the chronology of appearance of the islet T cell reactivity and by spread tolerance after GAD administration (167, 168)].

Another hypothesis involves the expression of a neoantigen at the β-cell surface secondary to the effect of a viral infection or a chemical. Finally, one may think of a bypass of anergized T cells by molecular mimicry after stimulation by an environmental factor (such as a virus or a cow's milk protein).

In all these hypotheses, an important role should be given to defective suppressor mechanisms amplifying the autoimmune response. Primary deficiency of regulatory T cells may give rise to autoimmune reaction as in the models of post thymectomy (and irradiated) models of autoimmunity. However, in view of the usually β-cell-restricted autoimmunity observed in human diabetes, it is unlikely that suppressor cell deficiency can by itself represent a sufficient factor to induce IDDM in most cases.

VIII. The β-Cell Lesion

A. Insulitis

The islets of Langerhans of recently diagnosed diabetic patients are infiltrated by mononuclear cells (insulitis) (169). These mononuclear cells include a majority of T cells (belonging to the two major subtypes CD4 and CD8, with apparently a predominance of CD8+ cells) and macrophages (170, 171). Some B cells may also be present. Fewer than 10% of β-cells persist 2–4 months after initiation of insulin therapy, as recently demonstrated by a pancreas biopsy study (258). This atrophy is selective for β-cells since other endocrine cells remain intact.

Studies of rodent models (217, 241, 259) have shown that destructive and invasive insulitis is preceded by periinsulitis (mononuclear cell infiltrate around the islets) and peripheral insulitis (lymphocytes at the islet periphery). Infiltrating T cells again include both CD4 and CD8 T cells, with signs of activation (IL-2 receptor expression). Transfer studies (217, 241) have shown that CD4 cells are the first cells to invade the islets. Interestingly, in the absence of CD4 cells (transfer of purified CD8 cells), CD8 cells do not migrate to the islets (241). Adhesion molecules (L-selectin) and very late antigen 4 (VLA 4) receptors may be involved in mediating leukocyte homing to the islets since insulitis and diabetes are inhibited in NOD mice by blocking these molecules by specific monoclonal antibodies (260). Immunohistological studies have shown that the infiltrating T cells express various cytokines such as IFNγ and IL-4, with a tendency for low IFNγ production and high IL-4 production in noninvasive insulitis contrasting with high IFNγ and low IL-4 levels in invasive insulitis (240), an interesting pattern which still requires confirmation. Importantly, there is no significant immunoglobulin deposition.

Studies of pancreatic sections in diabetic patients have revealed hyperexpression of class I and aberrant expression of class II MHC molecules at disease onset (170, 231). This important observation has been the subject of controversial findings in rodent models (232–235).

B. Inflammation vs. atrophy

It is important to kn w whether the β-cell dysfunction characteristic of IDDM is only due to β-cell destruction (atrophy) or can involve, in the early stages of clinical diabetes, a reversible functional inhibition (inflammation) leaving room for immunointervention at advanced stages of the disease. The latter is strongly supported by two sets of observations made in NOD mice. First, islets from recently diabetic NOD mice, which initially show low insulin production, regain part of their function when cultured *in vitro* in the absence of autologous T cells (261). Second, a single injection of an anti-TCR monoclonal antibody in NOD mice with established diabetes induces rapid normalization of glycemia (lasting throughout treatment) (262). This functional recovery must be distinguished from that observed in recently diagnosed diabetes after the start of intensive insulin therapy (263). The observed increase in C peptide production is then due to the release from glucotoxicity afforded by insulin.

C. Unique β-cell fragility

β-Cells appear to be particularly fragile cells, sensitive to a wide array of aggression. As mentioned above, hyperglycemia tends to reduce insulin secretion in addition to inducing peripheral insulin resistance. It is not known whether the relief from glucotoxicity explains the β-cell protection afforded by insulin therapy in NOD mice (201, 202), BB rats (203–205), and human prediabetics (206). It has been proposed that insulin could act by reducing the expression of β-cell autoantigens, but insulin may also prevent transient episodes of deleterious hyperglycemia.

Various cytokines can alter β-cells or even destroy them. This is particularly the case for IL-1 (264) and TNF (265), which are most active in combination. The effect of IL-1 is not totally β-cell-specific though, since α-cells are also affected and low IL-1 concentrations are only deleterious at supraphysiological glucose levels (266). In addition, administration of recombinant IL-1 induces hypoglycemia rather than hyperglycemia in normal and diabetic db/db and ob/ob mice (124) and prevents diabetes in NOD mice (267, 268). Similarly, TNFα, which shares many *in vitro* properties with IL-1, induces protection rather than acceleration of diabetes in NOD mice (269, 270) and BB rats (Tables 3 and 4). Other mediators could also intervene, possibly under cytokine control, such as nitrite oxide (NO), whose product is increased in NOD mouse islets (271) and whose inhibition by aminoguanidine delays the onset of diabetes in a transfer model (271).

It is not known whether β-cells from IDDM patients intrinsically show abnormally high fragility compared to those from healthy subjects. Pancreas and islet transplantation experiments mentioned above do not argue in this direction, since β-cells from non-diabetes-prone individuals appear to be fully sensitive to the effector mechanisms responsible for diabetes, as shown in NOD mice (118), BB rats (119), and humans (115–117).

D. Conclusions: the nature of pathogenic effector mechanisms (cell-mediated cytotoxicity or lymphokine effect ?)

Anti-islet cell autoantibodies are produced in large amounts in both rodent and human IDDM. There is no evidence, however, that these autoantibodies are pathogenic, even in the case of those directed at β-cell surface determinants. As just mentioned, no immunoglobulin deposits are found in islets. The disease cannot be transferred by serum of affected mice, whereas diabetes can be transferred by purified T cells in NOD recipients, even when the latter have been rendered unable to synthesize antibodies by perinatal anti-immunoglobulin M monoclonal antibody treatment (272).

T cells are beyond any doubt the main β-cell aggressors. Diabetes can be transferred to nondiabetic syngenic animals by purified T cells from diabetic NOD mice (142, 145) or BB rats (143) or T cell clones (144, 146) derived from diabetic NOD mice. Furthermore, selective T cell elimination by an anti-TCR monoclonal antibody normalizes hyperglycemia in diabetic NOD mice, as previously mentioned (262).

In contrast, there is still great uncertainty as to the intimate mechanisms of T cell-mediated aggression toward β-cells. Direct antigen-specific CD8+ T cell-mediated cytotoxicity is a logical hypothesis, since CD8 T cells are predominant in human IDDM-associated insulitis (170, 171). Additionally, CD8+ T cells are necessary to transfer diabetes to fully immunoincompetent irradiated or neonatal NOD mice (9, 10, 142, 146, 163) and BB rats (164). Also, NOD mice backcrossed with CD8 T cell-deprived mice whose MHC class I genes have been inactivated by homologous recombination do not develop diabetes (273). There is some evidence that CD8 T cells from diabetic patients and animals lyse β-cells (146, 274) but these results have been difficult to reproduce. CD8 T cells expressing the cytolytic mediator perforin are found in NOD mouse insulitis (275), but this mediator is found in most cytotoxic cells and not exclusively in antigen-specific cytolytic T lymphocytes. CD8 T cells have also been shown to inhibit insulin release by islet cells cultured *in vitro* (276), but the interpretation is complicated by the absence of MHC restriction in this model.

Diabetes can be transferred to young NOD mice by CD4 T cell clones alone (144, 146), even after administration of an anti-CD8 monoclonal antibody to rule out any involvement of host CD8 T cells (10, 277). This observation is at variance with previously mentioned evidence that CD8+ T cells are necessary for diabetes transfer. Perhaps young NOD mice (3–4 weeks) used for T cell clone transfer have some CD8+ T cells (even after anti-CD8 antibody treatment) that cooperate with the CD4 T cell clones. CD8 T cell clones have not proven capable of transferring the disease (146) but the addition of polyclonal CD8+ T cells from diabetic mice accelerates diabetes transfer by CD4+ T cell clones in irradiated recipients (146).

T cells could also intervene by secreting various lymphokines that can be directly toxic to β-cells or attract in the pancreas and activate other cell types such as monocytes, macrophages, and eosinophils all found in insulitis. These cells could in turn produce β-cell-toxic mediators such as IL-

1 or TNF to which, as mentioned above, β-cells are exquisitely sensitive. The prevention of diabetes btained in rodent models by treatment with antioxidants, desferrioxamine, or nicotinamide (Table 3) fits with this hypothesis, suggesting the pathogenic role of free radicals and, possibly, nitric oxide.

Such a role of lymphokines, known to be primarily produced by CD4+ T cells (rather than CD8+ T cells), is supported by the already mentioned capacity of CD4+ T cell clones to transfer diabetes (144, 146, 277) and the recurrence of diabetes after transplantation of MHC-incompatible islet grafts in NOD mice (118) or BB rats (119) in conditions excluding allogeneic rejection (prior islet culture *in vitro*): cytotoxic T lymphocytes cannot exert their activity against an MHC-incompatible target because of the MHC restriction of antigen recognition by T cells. It is also interesting to note that anti-CD4 monoclonal antibodies prevent recurrence of diabetes in islets grafted in NOD mice, whereas anti-CD8 monoclonals do not (118); however, this must be interpreted with caution since, in another model, both anti-CD4 and anti-CD8 monoclonals prevent cyclophosphamide-induced diabetes (our unpublished observation). The interpretation of these contradictory data should perhaps take into account the fact that when administered several days before grafting (as performed in the experiments just mentioned) (118), anti-CD4 monoclonals can induce long-term anti-islet unresponsiveness, which anti-CD8 monoclonals cannot (278).

Finally, the question of the respective involvement of CD4+ T cell-produced lymphokines and of CD8+ T cell-mediated cytotoxicity remains open, since none of the arguments supporting the role of one or the other provides absolute proof. The problem is complicated by the helper function of CD4+ T cells for CD8+ T cell differentiation. Alternatively, IFNγ produced by CD8+ T cells could enhance CD4+ T cell action. In conclusion, one may reasonably assume from data presented above that both subsets are needed for diabetes since elimination of either subset can prevent diabetes in NOD mice and BB rats. It is still difficult to say which cell exerts the central effector function and how each cell type regulates the other.

Attention should also be given in this context to the possible cytotoxic activity of natural killer (NK) cells and lymphokine-activated killer (LAK) cells that exert antigen-nonspecific cytotoxicity activated by lymphokines. There is some evidence in BB rats that such cells could play a significant role (176).

IX. Clinical Implications

The data and concepts discussed above have already generated a number of clinical applications and hold exciting prospects.

A. New appreciation of disease heterogeneity

When genetic factors and immune mechanisms are better defined, a new classification of diabetes mellitus will undoubtedly be formulated, distinguishing autoimmune diabetes from nonautoimmune diabetes.

Autoimmune diabetes will cover most (but not necessarily all) patients currently listed as having type 1 diabetes. It will also include the large number of patients with NIDDM due to a slow autoimmun anti-β-cell reaction. These patients are recognized by the presence of ICAs and the predisposing alleles DR3 and/or DR4. The proportion of slow type 1 among NIDDM patients reaches 10–15% according to studies (279–283). Identification of these patients, for example by ICA screening of NIDDM patients, is clinically important because of the possibility of early insulin therapy, which eventually becomes necessary in most of these patients after a long period of poor metabolic control (279, 281, 282).

A special place should be reserved for diabetes due to the direct cytolytic effect of viruses on β-cells [*e.g.* rubella (101)] and toxic agents [*e.g.* pentamidine (127)], even if the involvement of the immune system cannot be ruled out in these cases.

Finally, attention must be paid to nonDR3-nonDR4 fully insulin-dependent diabetics. The level of ICAs and sensitivity to cyclosporine are lower in these patients (67), who could represent an interesting etiological subgroup.

In fact, the question must be raised of the extent of IDDM heterogeneity. One may be lured by the study of the NOD mouse and the BB rats which, as mentioned above, represent only a single individual produced in multiple copies. The etiological role of multiple factors (genetic and environmental) is firmly established, but it is difficult to say whether all these factors intervene in a single patient or whether a limited number of them is involved in various combinations in individual subjects explaining the disease heterogeneity.

B. Predicting diabetes

We have seen that ICAs (and other islet-reactive autoantibodies) can be detected several years before the clinical onset of diabetes (284). These immunological markers, combined with the identification of predisposition genes (HLA and non-HLA genes), allow a fairly precise prediction of the disease risk in families of diabetic patients [~80% at 5 yr (see reviews in Refs. 285–289)]. ICAs and anti-insulin autoantibodies appear to be the best predictive markers at present. Anti-GAD and β-cell-restricted ICAs (which essentially include anti-GAD antibodies) appear to show a weaker association with diabetes onset and could even be a marker of protection (196, 289). One must realize, however, that genetic prediction will never exceed the concordance rate in identical twins (35–40%) and that HLA typing will never exceed the concordance rate in HLA-identical siblings (10–15%). Autoantibodies (whatever the test used) are absent in 15–20% of patients with recent-onset diabetes. Perhaps this gap could be filled by T cell assays, but none are yet operational. The complementary use of metabolic tests [assays of precocious insulin secretion following glucose infusion (284)] has not proven very informative, because of the high variability of the response in normal subjects and the late occurrenc of interpretable anomalies (only a few months before the onset of insulin dependency). The size of most families in Western countries being small, it is less likely that a prediabetic subject will have a diabetic sibling, and genetic and immunological tests are less efficient in the general population than within

affected families. All these limitations call for renewed research efforts to provide reliable prediction to the degree required for immunotherapy.

C. Immunotherapy

Immunotherapy can be used in human IDDM at three different stages of the disease.

Prediabetes without insulin requirement or even metabolic abnormalities after glucose infusion (to be distinguished from subjects who have all predisposing genes but in whom there is no evidence whatsoever of the initiation of the anti-β-cell autoimmune response). This is the ideal situation since a large fraction of the β-cell mass is still likely to be present and there are strong indications that the autoimmune response is more sensitive to immunointervention at this stage than later on. Unfortunately, only insulin prophylaxis has so far had any activity at this stage (206). Nicotinamide is being tested on the basis of suggestive nonrandomized preliminary studies (290–292).

Preclinical diabetes, where metabolic abnormalities are sufficiently marked to be detected by provocation tests but not to induce an insulin requirement. Slow type 1 diabetes can be placed in this category.

Overt diabetes, defined by insulin dependence. Immunointervention may still be efficacious at this stage, inasmuch as it is started within 6–8 weeks after the initiation of insulin therapy. It should be realized, however, that only a few β-cells are left at this time and one cannot expect a complete and long-term recovery of β-cell function at this stage. In this case, the objective is limited to preservation of the remaining β-cell mass (with possible improvement of β-cell function due to the action on local immunologically mediated inflammation). Even in cases where insulin cannot be withdrawn, a significant improvement of metabolic control may result due to the better efficacy of endogenously produced insulin in response to glucose stimulations than that of fixed insulin injections.

The large array of methods and products that have been successfully used in animal models have already been discussed (Tables 2 and 3). It must be stressed, however, that most of these interventions were applied early in the natural history of the disease, at a phase of "prediabetes" that is difficult to detect reliably in man. In addition, there are ethical problems involved in chronic treatment of young, apparently healthy, subjects. Hence the interest in products active on established diabetes (cyclosporin, monoclonal antibodies) and even more in products inducing long-term unresponsiveness (tolerance) without the need for continuous treatment. This objective has recently proven feasible in NOD mice, with polyclonal antilymphocyte sera (278), and both anti-CD4 (278, 293) and anti-CD3 (294) monoclonal antibodies. The mechanism of the tolerance induction in these experiments is not known but could involve stimulation of regulatory cells (TH2?) by T cells *in situ* under the cover of the anti-T cell antibody. Alternative experimental approaches to antigen-specific immunotherapy include peptide therapy (autoantigen peptide analogs binding to MHC molecules) (295) and intrathymic islet grafting, in an attempt to induce n gative selection of islet-reactive T cells (228, 229, 296). One should also mention the attempt to induce specific unresponsiveness (tolerance) in young (3- to 6-week-old) NOD mice using insulin given orally (200) or GAD given either intravenously (167) or intrathymically (168). It is interesting that in the oral insulin model the hypothesis has been put forward that tolerance to the introduced autoantigen leads to spread tolerance toward other β-cell autoantigens, possibly by local production of immunosuppressive cytokines such as TGFβ.

Therapeutic trials in human IDDM have as yet been limited to a small number of compounds, essentially in recent-onset diabetes. Two drugs have proven efficacious in rand mized studies: cyclosporin (*vs.* a placebo) (147, 148, 297) and azathioprine in association with steroids (298). One trial using low dose azathioprine (2 mg/kg/day) alone did not show any effect (299). In any case, the remission induced by these two agents was not indefinite (1–3 yr) (300) due to the occurrence of insulin resistance (301, 302) and to the autonomous nonimmunologically mediated deterioration of the remaining β-cell population induced by persistent hyperglycemia (glucotoxicity). However, one cannot exclude the persistence in these patients of an ongoing anti-β-cell autoimmune response since insulin resistance and glucotoxicity are not sufficient in the majority of type 2 diabetics to induce progressive β-cell destruction. Also, the rate of remission was no higher than 50%, and immunosuppressive therapy could not be stopped without rapid relapse.

Based on animal model data, three directions are being taken to circumvent these difficulties: 1) earlier therapy, based on prediction tests and using nontoxic drugs; 2) more acute intervention to improve efficacy and rapidity of action over the relatively slow-acting conventional immunosuppressive drugs [*e.g.* with IL-2/toxin conjugates (149)]; and 3) tolerance induction with either (oral or intravenous) autoantigen administration or monoclonal antibodies.

These approaches are being complemented by better usage of optimized insulin therapy and strict selection of patients for clinical trials.

X. Conclusions and Summary

IDDM is unquestionably an autoimmune disease, as reflected by the presence of β-cell-reactive autoantibodies and T cells, T cell-mediated transfer of the disease in nondiabetic mice, rats, and humans, and disease sensitivity to immunosuppressive therapy. T cells are predominantly, if not exclusively, involved in creating the islet lesions that lead to β-cell atrophy after a stage of reversible inflammation. A full understanding of the disease pathogenesis will require a better definition of the nature of the triggering and target autoantigen(s) and of the effector mechanisms (cytokines, cytotoxic cells?).

Much less information is available on the etiology than on the pathogenesis. Genetic factors are mandatory and the involvement of predisposition genes (HLA and non-HLA) is now being unravelled. The modulatory role of environmental factors is demonstrated by the high disease discordance rat in identical twins and by experimental data showing positive

and negative modulation of the disease by a number of agents, notably infectious agents and food constituents. It is not clear, however, whether a given environmental factor, *e.g.* a precise virus or a cow's milk component, plays a real eti logical role in a selected genetic background. IDDM thus appears as a multifactorial disease. It is not known, however, whether all factors intervene concomitantly in a given individual or separately in subsets of patients, explaining the clinical heterogeneity of the disease.

The mechanisms underlying the loss of tolerance to self β-cell autoantigen(s) are still unknown. Defective intrathymic negative selection of autoantigen-specific autoreactive T cell clones is unlikely. Breakdown of T cell anergy could occur according to various mechanisms, including aberrant expression of MHC molecules and molecular mimicry. Defective suppressor T cell function, perhaps related to TH1/TH2 imbalance, probably intervenes by amplifying the anti-β-cell autoimmune response whatever its triggering mechanism.

Before putative etiological agents are identified, one must base immunotherapy on nonantigen-specific agents. Results recently obtained in NOD mice indicate that the goal of nontoxic long-lasting immune protection from the disease is feasible if treatment is started early enough. In some cases (anti-T cell monoclonal antibodies), it appears that specific unresponsiveness can be induced. This double strategy (early intervention, tolerance induction) is the main challenge for immunodiabetologists. They must convince clinical diabetologists, the patients, and their families that immunoprevention of the disease will only be achievable if research on prediction and immunotherapy proceeds hand in hand. Prediction programs are difficult to run without proposing a safe and potentially efficacious preventive therapy, and the search for therapy cannot be successful without access to prediabetics or patients with preclinical diabetes, who can only be identified in prediction clinics. Hopefully this review will contribute in a modest way to generating the necessary faith in the future of immunoprevention of the disease, which could eventually lead to its eradication.

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Prevention of diabetes in the NOD mouse: implications for therapeutic intervention in human disease

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The prevention of insulin-dependent diabetes (IDD) in humans remains an elusive goal, despite the broad spectrum of therapeutic interventions that prevent the development of IDD in the non-obese diabetic (NOD) mouse. Can an animal model in which spontaneous autoimmune pathology is interrupted so easily serve as an archetype for the design of clinical trials aimed at the prevention of IDD in humans? In this article, Mark Bowman, Edward Leiter and Mark Atkinson review the intervention strategies that prevent IDD in the NOD mouse and indicate why these studies may well be relevant to the prevention of IDD in humans.

Many key features of human insulin-dependent diabetes (IDD) are reflected in the non-obese diabetic (NOD) mouse: the development of insulitis, whereby pancreatic islets of Langerhans are infiltrated by lymphocytes that are selectively cytotoxic to the insulin-producing β cells; the inheritance of particular major histocompatibility complex (MHC) class II alleles, representing the major component of genetic susceptibility; the transmission of IDD by hematopoietic cells in bone marrow; and the T-cell dependence of disease pathogenesis[1-5] (Table 1). The origin, genetics and immunological characteristics of the NOD strain, as well as the ability of environmental manipulations to effect the diabetogenic processes in these mice, have recently been reviewed[3,6,7]. For reasons of brevity, the reader is referred to these reviews for references to much of the information described below.

There are relatively few characteristic differences between human and mouse disease. The presence of a high percentage of T cells (both $CD4^+$ and $CD8^+$ subsets) in NOD lymphoid tissues and peripheral blood distinguishes NOD mice from humans with IDD and from the severely T-lymphopenic diabetes-prone BB/Wor rat, which also spontaneously develops autoimmune IDD (Ref. 8). NOD mice do not display the severe diabetic ketoacidosis characteristic of untreated human patients with IDD, perhaps due to an enhanced ability of mice to metabolize blood ketones to lactate. In addition, NOD mice exhibit a pronounced female gender bias for disease susceptibility that is not observed in BB rats or in humans. While the specific reasons for this difference are unclear, female mice are known to be stronger immunological responders to exogenous stimuli than males and it has been demonstrated that the lower incidence of IDD in NOD males is partially regulated by gonadal sex steroids. Finally, the pathogenesis of IDD in NOD mice is associated with expression of endogenous defective retroviruses[7] in β cells, a feature that has not been described previously in the β cells of insulitic islets from BB rats or humans.

One major distinction must be considered when comparing IDD etiopathogenesis in humans and NOD mice. Given the genetic heterogeneity within the human population, the development of IDD is likely to reflect heterogeneous mixtures of susceptibility genes whose penetrances are responsive to different thresholds of intragenic and environmental influences. NOD mice inherit the same gender-specific set of susceptibility genes, a consequence of over 50 generations of sibling matings. The penetrance of these genes can be analysed under constant, well-defined environmental conditions of diet, temperature and exposure to pathogenic agents. Consequently, the natural history of IDD development in a well-maintained NOD colony is quite predictable. Indeed, an intervention study in NOD mice can be designed such that treatment is initiated at a presymptomatic stage prior to the occurrence of insulitis (up to 3 weeks postpartum), or before the onset of disease (four to eight weeks postpartum), at a time when considerable numbers of β cells are still intact. By contrast, the genetic and environmental heterogeneity associated with the natural history of IDD in humans is such that the age of disease onset is extremely broad and may occur at any time from the first years of life to well beyond 50 years of age. Given these complexities, it has been difficult for clinical investigators to develop simple diagnostic tools for the early identification of humans destined to develop IDD. For these reasons, studies to prevent IDD in NOD mice must be carefully analysed for their applicability to therapeutic intervention in human disease.

Table 1. Comparison of insulin-dependent diabetes in humans and NOD mice

Characteristic	Humans	NOD mice
Genetic predisposition (MHC class II linkage)	+	+
Complex polygenic control	+	+
Environmental effects on gene penetrance	Probable	+
Disease transmissible *via* bone marrow	+	+
T-lymphocyte-driven insulitic lesions	+	+
Leukocytic infiltrates found in other organs	Sometimes	+
Defective peripheral immunoregulation	+	+
Humoral reactivity to β cells	+	+
Endogenous retroviral genes expressed in β cells	−	+
Diabetic ketoacidosis if untreated	+	Mild
Gender bias	±	+
Successful intervention therapies	Ongoing	+

Role of genetics and the environment

The inheritance of susceptibility to disease in humans and NOD mice is polygenic. In NOD mice, homozygosity of the MHC class II region of the unique H-2^{g7} haplotype is necessary, but not sufficient, for development of overt IDD (Ref. 7). Destruction of sufficient numbers of β cells to produce persistent hyperglycemia and glycosuria, the clinical phenotype of IDD, requires a complex interaction with numerous other genes that are not linked to the MHC locus. Although the relatively high discordance rates for IDD in monozygotic twins suggest an important role for the environment in human IDD, an environmental influence has not been unequivocally proven[9]. By contrast, it has been clearly demonstrated that penetrance of diabetes-susceptibility genes in the NOD mouse is strongly influenced by agents in the extrinsic environment, including dietary components and microbial pathogens[10,11].

The protective effects of exposure to microbial pathogens is of considerable interest for two reasons. First, while there are epidemiological and anecdotal data suggesting that viruses may precipitate the autoimmunity that results in human IDD (Ref. 12), viral and bacterial infections have more often been reported to reduce rather than exacerbate the incidence of diabetes in the NOD mouse. These data contradict the paradigm of molecular mimicry, wherein a microbial antigen is sufficiently similar to self antigens to provoke a pathogenic cross-reactive autoimmune response. Second, although many current therapies for autoimmune disease involve immunosuppression, the effect of microbial challenge on diabetes in the NOD mouse presumably occurs through immunostimulation[7].

A recent analysis of worldwide NOD mouse colonies showed that the cumulative incidence of diabetes at 30 weeks is more variable (as well as lower) in males than in females[13]. Although some of the colony differences may be explained by genetic divergence amongst substrains of NOD mice separated from the original source colony, most of the differences appear to be environmentally driven. NOD males are particularly susceptible to modulation of diabetes development, since Caesarian transfer of pups from a conventional environment to a pathogen-free environment markedly increases the incidence of diabetes[14].

Developmental and functional defects have both been reported in the antigen-presenting cells (APCs) of NOD mice[15,16]. These defects appear to perturb presentation of self antigens in the course of tolerance induction. Some of these defects are associated with defective secretion of endogenous cytokines, including interleukin 1 (IL-1), IL-2 and IL-4 (Refs 17,18). It is possible that exposure to microbial pathogens may counterbalance these defects *via* elevation of inflammatory cytokine levels. Indeed, exposure of NOD mice to viral pathogens such as encephalomyocarditis virus (EMCV), lymphocytic choriomeningitis virus (LCMV) and murine hepatitis virus (MHV), or to bacteria (e.g. *Mycobacterium* and *Streptococcus*) and their components (e.g. complete Freund's adjuvant (CFA) and OK432), prevents and/or delays the onset of IDD (Refs 19–25). This may result from an upregulation of APC function by inflammatory cytokine release, since the IDD-protective effects produced by virological challenge to the immune system can be mimicked by treating young NOD mice with poly [I:C], a potent inducer of immune interferons[17]. The pleiotropic actions of the cytokine cascades that are initiated by treating NOD mice with viruses, bacteria and bacterial products result in the generation of an increased number of functional immunoregulatory cells (including T cells) capable of suppressing diabetogenesis[21–25].

Therapeutic interventions

Intervention strategies in the NOD mouse can alter the incidence of insulitis, spontaneous diabetes or cyclophosphamide-induced diabetes. However, it is important that the incidence of overt IDD, rather than insulitis, is used as an experimental readout in intervention studies, since insulitis is an uncertain predictor of IDD development. Hence, this review will only consider protocols that clearly alter the rate or total incidence of IDD development in colonies of NOD mice in which the investigators have demonstrated a high incidence of spontaneous or cyclophosphamide-induced diabetes in controls. Furthermore, diabetes-related studies in transgenic mice will not be discussed.

Despite limiting discussion to these stated criteria, successful interventions are remarkably abundant and diverse (Box 1). Indeed, the ease of manipulating IDD incidence in the NOD mouse attests to the utility of this model for dissecting the multiple components of a complex disease. The explanation for the success in identifying such a broad spectrum of factors capable of retarding or preventing IDD in NOD mice may be quite straightforward. Each mouse inherits the same set of diabetes-susceptibility genes and, by controlling the physical and biological environment in which these genes are expressed, investigators have established a longitudinal progression of immunological and endocrinological events leading to IDD (Refs 26,27). For example, in NOD females, pancreatic islet-infiltrating leukocytes are first observed at three to five weeks postpartum, whereas a significant decline in pancreatic insulin content is not detected until

approximately 12 weeks. The clinical presentation of hyperglycemia and glycosuria typically occurs at age 16–20 weeks in 50% of fully mature NOD females. The early infiltrates contain fewer T-cell effectors, as demonstrated by the finding that adoptive transfer of splenic T cells from young prediabetic mice into NOD–severe combined immunodeficiency (NOD–SCID) mice requires a longer period before onset of IDD than does transfer using an equal number of cells from diabetic donors[28].

Thus, a broad 'window' for therapeutic intervention exists between the time that mononuclear infiltrates first surround the pancreatic islets and the time that β-cell numbers fall below a level required to maintain normoglycemia. Therapeutic protocols that have been used successfully to interrupt the pathogenic process in NOD mice (summarized in Box 1) can be grouped into the following categories: (1) immunosuppression; (2) immunostimulation; (3) tolerance induction or placing β cells in a metabolic 'resting' state; (4) manipulation of the hormonal or dietary milieu; and (5) treatment with anti-inflammatory agents.

Immunosuppression

T-cell deficient NOD mice homozygous for either the nude (nu) or SCID mutation do not develop insulitis or diabetes. Furthermore, treatments that compromise the effector functions or viability of either the $CD4^+$ or $CD8^+$ T-cell subsets can retard or circumvent diabetes. Immunosuppressive agents capable of retarding or preventing IDD onset include cyclosporin A, FK506, rapamycin and deoxyspergualin[29,30]. Similarly, successful intervention studies employing an assortment of antibodies directed towards the T-cell surface also exist, including antibodies to the T-cell receptor (TCR) α and β chains, anti-lymphocyte serum, anti-CD3, anti-Thy 1.2, anti-CD4 and anti-CD8 (Refs 31–34). It appears that B lymphocytes are not required at the effector level, since adoptive transfer of NOD T lymphocytes into anti-μ suppressed neonatal recipients still results in diabetes. NOD mice do not express lytic complement activity due to a mutation in the *He* gene encoding C5a (Ref. 35).

It has been found that 'irrelevant' control monoclonal antibodies to certain TCR Vβ clonotypes that are not present in NOD mice unexpectedly reduce the incidence of disease. However, this protection may be mediated by nonspecific immunostimulation as a consequence of an antiglobulin immune response. Indeed, it is not uncommon for NOD mice to produce antibodies against the monoclonal antibodies being tested for therapeutic potency.

Immunostimulation

A striking characteristic both of NOD mice and patients with IDD is a markedly depressed autologous mixed lymphocyte reaction (AMLR), which in turn suggests a defect in peripheral immunoregulatory mechanisms. Defects in cytokine-elicited differentiation and maturation of APCs from the bone marrow of NOD mice may result in the inefficient presentation of self antigens and impair the tolerogenic capacity of these cells. These defects could explain why exposure of prediabetic NOD mice to a number of environmental pathogens imparts resistance to diabetes. Thus, certain environmental stimuli may upregulate APC function and (1) increase thymic deletion (or thymic or peripheral anergization) of autoreactive T-cell clones, or (2) potentiate the activation of immunoregulatory T lymphocytes in the periphery, or tolerize by a combination of these mechanisms. This concept is reinforced by the finding that chronic administration of a variety of cytokines, or single injections of potent immunomodulators that upregulate endogenous cytokine expression, also circumvent diabetes (Box 1). Indeed, many therapeutic manipulations may stimulate antigen processing and presentation by macrophages. Protection associated with some of these treatments, for example administration of IL-1 or IL-2, restores a more-normal AMLR, although chronic treatment with IL-4 does not.

Box 1. Therapies that prevent diabetes in the NOD mouse

Immunosuppression
T-cell functions: neonatal thymectomy, anti-lymphocyte serum, anti-Thy-1, anti-CD3, anti-CD4, anti-CD8, cyclosporin, FK-506.
Macrophage/APC functions: anti-complement receptor, silica, LDHV, anti-MHC class I, anti-MHC class II, blocking peptide for MHC class II, anti-IFN-γ.

Immunostimulation
Pathogenic viruses: LCMV, EMCV, MHV.
Cytokines or cytokine inducers: IL-1, TNF-α, IL-4, IFN-γ, poly [I:C], Con A.
CFA, BCG, OK432, heat-shock protein 65.

Tolerance induction
Bone-marrow transplantation.
Intrathymic islet transplantation.
Oral insulin.
Dendritic cells from pancreatic node.
Neonatal tolbutamide treatment.
Immunization with insulin or insulin B chain.

Manipulation of hormonal/dietary milieu
Gonadectomy.
Prophylactic insulin treatment.
Diazoxide.
Elevated temperature.
Semi-purified diets.

Anti-inflammatory agents
Nicotinamide, superoxide dismutase-desferrioxamine, vitamin E, aminoguanidine.

Abbreviations: APC, antigen-presenting cell; LDHV, lactate dehydrogenase virus; MHC, major histocompatibility complex; IFN-γ, interferon γ; LCMV, lymphocytic choriomeningitis virus; EMCV, encephalomyocarditis virus; MHV, murine hepatitis virus; IL, interleukin; TNF-α, tumor necrosis factor α; Con A, concanavalin A; CFA, complete Freund's adjuvant; BCG, Bacille Calmette Guérin.

Other immunological defects characteristic of NOD mice may also be ameliorated by immunostimulation. These include: subnormal lipopolysaccharide (LPS)-stimulated IL-1 secretion; subnormal secretion of IL-2 and IL-4 by splenic and thymic T cells, respectively; and depressed thymocyte responses to mitogenic stimulation.

Tolerance induction or β-cell 'rest'

A single injection of anti-CD3 monoclonal antibody into neonatal NOD mice is tolerogenic[36]. Tolerance has also been achieved through the selective destruction or self-inactivation of autoreactive T cells, without damaging the function of all T cells. Thymic deletion of autoreactive clones can be enhanced by intrathymic injection of islet cells into neonatal or adolescent NOD mice[37]. Similarly, the peripheral deletion of autoreactive clones has been achieved *via* vaccination with autoreactive T-lymphocyte lines[38], activated $CD4^+V\beta8^+$ T cells[39], and T-cell clones specific for a 65 kDa heat-shock protein[40]. Other protocols, such as the oral administration of insulin[41], or the injection of dendritic cells or splenocytes[42], may be more likely to induce regulatory tolerance or clonal anergy in T lymphocytes reactive to islet antigens.

Prophylactic insulin therapy prevents diabetes and the formation of insulitis in NOD mice[43]. Whether or not this mechanism is due to β-cell 'rest' (Ref. 44 and see below), or to the induction of tolerance, is unknown. According to the concept of β-cell rest, chronic treatment with diazoxide suppresses insulin secretion and reduces diabetes by causing the β cells to be less visible to the immune surveillance system, or less susceptible to inflammatory damage. However, a recent study demonstrated that a potent insulin-stimulator, tolbutamide, also reduced the incidence of IDD (Ref. 45). Furthermore, administration of insulin, either orally or intraperitoneally (i.p.), protects from diabetes and i.p. injections of the insulin B chain is as protective as intact insulin[46]. The similar effect of oral and i.p. administration of whole insulin argues that prophylactic insulin therapy protects *via* insulin tolerization, rather than through β-cell rest. Indeed, recent studies indicate that the IDD-protective effects of intrathymic or intravenous administration of recombinant glutamic acid decarboxylase (GAD-65) into NOD females during weaning are achieved by acquisition of T-cell tolerance to this candidate β-cell autoantigen[47,48]. Although stimulation in early life may not be applicable to the period of β-cell destruction, the concept of β-cell rest has also been questioned by the observation that neonatal glucose treatment in NOD mice reduces diabetes frequency and stimulates insulin secretion[49].

Treatments that may interfere with the presentation of antigenic peptides to T cells include the silica-mediated destruction of macrophages[50], as well as the administration of antibodies against mouse MHC class II I-A molecules[51], and 'blocking' peptides[52] that compete for binding to $I\text{-}A^{g7}$. However, antibody blocking of macrophage complement receptors probably protects by preventing the recruitment of these cells into insulitic lesions. The protection against diabetes that results from infection with lactate dehydrogenase virus (LDHV) may also be mediated *via* effects on the macrophage population.

Manipulation of hormonal/dietary milieu

It has been found that the occurrence of diabetes in mouse colonies with a low incidence of spontaneous diabetes in males increases after castration and that androgen treatment protects females[53,54]. The protective effect of semi-purified diets may be mediated by the alteration of cytochrome p450 activities in the liver which, in turn, may control the level of active androgens and estrogens in tissues (E.H. Leiter *et al.*, unpublished). The reduced incidence of diabetes that is observed when temperatures are increased may relate to a decreased intake of diabetogenic natural-ingredient diets. Although bovine milk proteins have sometimes been associated with the diabetogenic properties of natural-ingredient chow diets, the association was not reproduced in NOD colonies with a high incidence of spontaneous diabetes.

Anti-inflammatory agents

One strategy for preventing diabetes in NOD mice is based on the assumption that destruction of β cells results from the release of oxygen radicals by macrophages. Administration of oral nicotinamide, superoxide dismutase-desferrioxamine and vitamin E have the effect of reducing such oxidative processes in β cells[55,56]. Other preventative therapies arise from the suggestion that IL-1 and other inflammatory cytokines induce islet β cells to produce toxic levels of nitric oxide, resulting in cellular suicide. A recent report claiming prevention/delay in the adoptive-transfer model of IDD using aminoguanidine (a competitive inhibitor of nitric oxide synthase) supports a role for nitric oxide production in the pathogenesis of β-cell destruction[57].

Human trials for IDD prevention

To date, the major research effort directed at prevention of IDD in humans has focused on the effects of nonspecific immunotherapy administered at the time of diagnosis of IDD, such as cyclosporin and azathioprin. Pharmacological agents such as these have had an extensive history of use in a variety of clinical situations, for instance in organ transplantation and rheumatoid arthritis. Multiple investigations have demonstrated that treatment of new-onset IDD patients with these agents leads to the ability of some patients to produce insulin (i.e. C-peptide) over prolonged periods, and a number of anecdotal reports have described patients who have undergone clinically significant remissions of disease[58,59]. Unfortunately, in most individuals, the effect of these drugs was not lasting enough to be of clinical significance and reports of permanent remissions from IDD were rare.

Human IDD is preceded by a long presymptomatic period – suggesting that the clinical symptoms of disease arise only after the destruction of the majority of β cells. Many researchers believe that effective methods of disease prevention will require therapy at an earlier, preclinical asymptomatic stage. This preclinical period

can be identified effectively through autoantibody markers of anti-islet immunity, with a majority of studies to date utilizing the islet-cell cytoplasmic autoantibody[1,2]. However, the use of immunosuppressive agents in asymptomatic individuals is controversial. Problems associated with the long-term use of immunosuppressive agents include toxic side-effects, such as nephrocytotoxicity, and the increased frequency of developing viral infections and cancer (e.g. B-cell lymphomas)[60]. These risks have led to a search for less-aggressive interventions that prevent IDD without impairing normal immune functions. Although most of this research has involved studies of diabetes interventions in the NOD mouse, a number of these studies are now being considered for human clinical trials. Prophylactic insulin therapy prevents diabetes in each of the rodent models for IDD (BB/Wor rats and NOD mice) and, in NOD mice, the therapy also prevents insulitis[41]. Prophylactic insulin has recently undergone pilot trials in humans and shows promising preliminary results[61].

Anti-inflammatory agent inhibitors have also shown promise in preventing IDD in NOD mice. For this reason, oral nicotinamide has been used in two pilot studies to prevent IDD in asymptomatic individuals who have increased risk for IDD, and preliminary results indicate success[62]. The beneficial effects of this drug may derive from prevention of β-cell damage by oxygen or nitrogen (e.g. nitric oxide) free radicals. However, although nicotinamide is commonly viewed as a safe drug, higher doses than those used in human clinical trials have reportedly resulted in liver dysfunction[63].

Another interesting strategy that is being considered for clinical trials in humans is the oral induction of tolerance to islet-cell proteins that have been implicated as autoantigens in anti-islet-cell immunity. Indeed, the onset of diabetes was both attenuated and protracted in NOD mice fed with porcine insulin[42]. Although the mechanism for this effect is unresolved, it may be mediated by the generation of regulatory T cells that are reactive to insulin. After migrating to the pancreatic islets and encountering insulin, these lymphocytes may secrete regulatory cytokines such as IL-10 and transforming growth factor β (TGF-β), and thereby suppress bystander autoimmune responses. Recent studies in NOD mice have shown that parenteral administration of insulin is as effective as oral administration in preventing IDD (Ref. 46). Given the paucity of side effects, as well as the targeted nature of these antigen-based therapeutic approaches, the oral and/or parenteral administration of either GAD (Refs 47,48) or insulin may have great applicability to the prevention of IDD in humans.

The prevention of IDD in rodents can also be achieved by immune-enhancement therapy. This approach, which at first appears paradoxical, is based on the hypothesis that the essential problem underlying IDD is the inability to maintain peripheral immunological tolerance to the pancreatic islets actively. Thus, any number of nonspecific immunization events may enhance general tolerogenic mechanisms sufficiently so as to prevent disease. Indeed, the administration of carefully timed doses of CFA has been shown to prevent diabetes in NOD mice[64]. Similarly, treatments with recombinant IL-1, IL-2, tumor necrosis factor α (TNF-α) and poly [I:C] can all prevent diabetes[65]. Human trials that will test this hypothesis by actively immunizing individuals with Bacille Calmette Guérin (BCG) are planned.

Finally, considerable interest has been generated by the observation that intrathymic transplantation of islet cells at birth in rodent models of IDD both prevents insulitis and diabetes[37,41,66]. T-cell tolerance is mediated, in part, by interactions between maturing thymocytes and self antigens presented by thymic stromal cells. The beneficial effect of intrathymic transplantation may be the result of the specific modulation of diabetogenic T cells that are forced to mature in a thymic microenvironment enriched for islet antigen. Once the autoantigen(s) that elicits human IDD is identified, autoantigen-immunization in early life may result in tolerance to the antigen and prevention of IDD. Thus, a method for the induction of specific tolerance to islet-cell antigen in humans would appear to be promising.

Conclusions

The NOD mouse has provided a model system to study not only the pathogenesis and natural history of a disease that is similar to human IDD, but also a means with which to test intervention protocols that could be used to prevent the disease in humans. Indeed, studies in NOD mice also indicate that immunostimulation may be a more viable means of keeping the prediabetic patient non-diabetic than immunosuppression.

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Treatment of NOD Diabetes with a Novel P ptide of the hsp60 Molecule Induces Th2-type Antibodies

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A peptide from the sequence of hsp60 molecule, designated p277, has been shown to be functionally involved in modulating the development of autoimmune diabetes in the NOD mouse: administration of p277 to NOD mice can arrest the diabetogenic autoimmune process, even when far advanced. Is p277 the only hsp60 peptide able to modulate the disease? We mapped T cell responses to peptides spanning the mouse hsp60 molecule and identified an immunogenic peptide, designated p12, that is also functional in arresting NOD diabetes. Although no spontaneous T cell reactivity to p12 could be detected in NOD mice, subcutaneous administration of 100 μg of p12 in mineral oil to 10-week-old female NOD mice, similar to treatment with p277, significantly prevented progression of the disease. Administration of other immunogenic peptides was not effective. A peptide from the glutamic acid decarboxylase (GAD65) sequence, GADp35, and a peptide from the mycobacterial hsp60 molecule did not influence the development of diabetes. The effectiveness of hsp60 peptides p12 and p277 was associated with the induction of antibodies to the peptides of the IgG1 and IgG2b isotypes, antibodies which appear to be regulated by anti-inflammatory cytokines. There was a negative correlation between the amounts of antibodies induced by the hsp60 peptides and the level of blood glucose. Thus, more than one peptide of the hsp60 molecule can be used to inhibit the development of NOD diabetes, and the effect of peptide therapy appears to be associated with the induction of specific antibody isotypes.

Introduction

Insulin-dependent diabetes mellitus (IDDM) develop[illegible] spontaneously in female NOD mice has been [illegible]ciated with immune reactivity to a variety of self-antigens (reviewed in [1]). Notable among these antigens is the p277 peptide from the sequence of the mammalian 60 kDa heat shock protein (hsp60) molecule, residues 437–460. Prediabetic NOD mice manifest spontaneous, diabetogenic T cell responses to hsp60 and to the human [2] or mouse variants of the p277 peptide [3]. The mouse and human peptides differ by one amino acid and are immunologically cross-reactive [3]. Some non-diabetes-prone strains of mice, such as C57BL/6, develop transient hyperglycemia and insulitis when immunized to p277 covalently conjugated to a foreign immunogenic carrier molecule [4]. Also, mice of the C57BL/KsJ strain develop spontaneous T cell responses to hsp60 and to p277 after treatment with a very low dose of the β-cell toxin streptozotocin (STZ), that induces autoimmune diabetes [5].

In addition to being involved in the expression of the disease, peptide p277 appears to be functional in arresting the autoimmune process: subcutaneous administration of p277 in incomplete Freund's adjuvant (IFA; mineral oil) led to arrest of disease progression in young NOD mice [2] or in 12–17-week-old NOD mice with advanced insulitis [6, 7]. Both the human [6, 7] and mouse [3] variants of p277 were effective. NOD mice transgenic for the mouse hsp60 gene on an MHC class II promoter showed down-regulation of their spontaneous T cell proliferative response to p277 and a significant proportion of the mice were spared the development of diabetes [8]. Moreover, administration of p277 to C57BL/KsJ mice aborted the development of autoimmune diabetes in mice that had earlier received a very low dose of STZ; treatment of these mice with an immunogenic peptide of the GAD65 molecule was not effective [9]. The response to treatment with p277 in the STZ model was associated with the induction of antibodies to p277 of the IgG1 and IgG2b isotypes [9]. Since mouse antibodies of the IgG1 isotype are induced by the cytokine IL-4 [10], these findings are compatible with the

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hypothesis that the development of type 1 diabetes may be influenced by the Th1-Th2 balance of the autoimmune response [11]. Indeed, effective treatment of 12-week-old NOD mice with peptide p277 was associated with the induction of antibodies of the IgG1 and IgG2b isotypes, which appeared to mark a shift in the cytokine profile of the anti-p277 T cell population from Th1-type secretion of IFN-γ to Th2-type secretion of IL-4 and IL-10 [12].

The arrest of murine diabetes, spontaneous and STZ-induced, by treatment with p277 raises a number of questions. Is p277 the only peptide of hsp60 that can be used to treat the disease process? Is the effectiveness of p277 in arresting the disease related to the existence of spontaneous T cell reactivity to p277? Will any peptide for which there is spontaneous T cell reactivity be effective in NOD diabetes, irrespective of whether the peptide is self- or foreign? Will other effective peptides also induce Th2-type antibodies in NOD mice? To investigate these questions, we mapped the T cell proliferative response of NOD mice to overlapping peptides spanning mouse hsp60 and selected a peptide, designated p12 (residues 166–185), that was immunogenic for NOD T cells. In contrast to p277, however, we could not detect spontaneous T cell proliferation to p12 in prediabetic NOD mice. We treated 10-week-old NOD mice with p12, p277, an immunogenic GAD65 peptide, GAD-p35, or with the foreign mycobacterial hsp60 peptide, MT-p278, for which our NOD mice manifest spontaneous T cell proliferation [12]. Only the hsp60 peptides p12 and p277 were effective in inhibiting diabetes, and only these peptides induced high titers of specific antibodies of the IgG1 and IgG2b isotypes. Thus we may conclude that more than one domain of hsp60 can be effective in arresting NOD diabetes, that pre-existing spontaneous T cell reactivity to a peptide may not be a prerequisite for a therapeutic response, and that other self- or foreign peptides immunogenic for NOD T cells may not be effective.

Table 1. *Overlapping peptides of the mouse hsp60 molecule used for screening*

Peptide number	Position	Sequence
p1	1–20	MLRLPTVLRQMRPVSRALAP
p2	16–35	RALAPHLIRAYAKDVKFGAD
p3	31–50	KFGADARALMLQGVDLLADA
p4	46–65	LLADAVAVTMGPKGRTVIIE
p5	61–80	TVIIEQSWGSPKVTKDGVTV
p6	76–95	DGVTVAKSIDLKDKYKNIGA
p7	91–110	KNIGAKLVQDVANNTNEEAG
p8	106–125	NEEAGDGTTTATVLARSIAK
p9	121–140	RSIAKEGFEKISKGANPVEI
p10	136–155	NPVEIRRGVMLAVDAVIAEL
p11	151–170	VIAELKKQSKPVTTPEEIAQ
p12	166–185	EEIAQVATISANGDKDIGNI
p13	181–199	DIGNIISDAMKKVGRKGVI
p14	195–214	RKGVITVKDGKTLNDELEII
p15	210–229	ELEIIEGMKFDRGYISPYFI
p16	225–244	SPYFINTSKGQKCEFQDAYV
p17	240–259	QDAYVLLSEKKISSVQSIVP
p18	255–275	QSIVPALEIANAHRKPLVIA
p19	271–290	LVIIAEDVDGEALSTLVLNR
p20	286–305	LVLNRLKVGLQVVAVKAPGF
p21	301–320	KAPGFGDNRKNQLKDMAIAT
p22	316–335	MAIATGGAVFGEEGLNLNLE
p23	331–350	NLNLEDVQAHDLGKVGEVIV
p24	346–365	GEVIVTKDDAMLLKGKGDKA
p25	361–380	KGDKAHIEKRIQEITEQLDI
p26	376–395	EQLDITTSEYEKEKLNERLA
p27	391–410	NERLAKLSDGVAVLKVGGTS
p28	406–425	VGGTSDVEVNEKKDRVTDAL
p29	421–440	VTDALNATRAAVEEGIVLGG
p30	436–455	IVLGGGCALLRCIPALDSLK
p31	451–470	LDSLKPANEDQKIGIEIIKR
p32	466–485	EIIKRALKIPAMTIAKNAGV
p33	481–500	KNAGVEGSLIVEKILQSSSE
p34	496–515	QSSSEVGYDAMLGDFVNMVE
p35	511–530	VNMVEKGVIDPTKVVRTALL
p36	526–545	RTALLDAAGVASLLTTAEAV
p37	541–560	TAEAVVTEIPKEEKDPGMGA
p38	556–573	PGMGAMGGMGGGMGGGMF

Materials and Methods

Mice

Female NOD/Lt mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The onset of diabetes occurs at about 13–15 weeks and the incidence of diabetes in these mice is 80% or greater by the age of 32 weeks, provided that the mice are maintained under specific pathogen-free conditions. Inbred male NOD mice, 8–10 weeks of age, were supplied by the animal-breeding center of this institute. The breeding nucleus was the gift of Dr E. Leiter of the Jackson Laboratory.

Antigens

Peptides were synthesized in the Department of Organic Chemistry of the Weizmann Institute of Science using an automated multiple peptide synthesizer (Abimed model AMS 422; Langenfeld, Germany) following the company's protocols for N-α-fluorenylmethoxycarbonyl (Fmoc) synthesis. Peptides were purified by reversed phase HPLC on a semi-preparative C_8-column (Lichrosorb RP-8, 7 μm, 250×10 mm, Merck, Darmstadt, Germany). Elution of peptides was achieved by linear gradients established between 0.1% trifluoracetic acid in water and 0.1% trifluoracetic acid in 75% acetonitrile in water (v/v). The purity of the single peptide products was ascertained by analytical reversed-phase HPLC and amino acid analysis. Table 1 shows the sequences of the 38 overlapping 20 mer peptides, with overlaps of five amino acids, that we used to span the mouse hsp60 sequence. Peptide p277 corresponds to the 437–460 sequence of mouse hsp60 and is not represented intact in the 20 mer spanning peptides. Peptide p277 is substituted at positions 6 and 11 with valine (V) in

Table 2. *Amino acid sequences of peptides used for treatment*

Peptide	Sequence
p277	VLGGGVALLRVIPALDSLTPANED
p12	EELAQVATISANGDKDIGNI
MT-p278	EGDEATGANIVKVALEA
GAD-p35	SRLSKVAPVIKARMMEYGTT
p38	PGMGAMGGMGGGMGGGMF

place of the cysteine (C) in the native sequence [7, 9, 12]. Substitution of the two C residues by V greatly enhances the stability of the peptide without affecting its immunological activity: the V-substituted peptide is completely cross-reactive with the native peptide by T cell and antibody assays (in preparation). Peptide GAD-p35 is from the GAD65 molecule (524–543). A non-immunogenic peptide, p38, from hsp60 (556–573) was used as a negative control. Peptide MT-p278 is from the sequence of mycobacterial hsp60 (431–447). NOD mice housed in our facilities manifest spontaneous T cell proliferative responses to MT-p278 [12]. The amino acid sequences of the peptides used in the functional studies are shown in Table 2.

Peptide mapping and T cell proliferation

For peptide mapping, the 38 peptides covering the mouse hsp60 sequence (Table 1) were used to immunize groups of three male NOD mice with pools of four peptides, 25 µg each, emulsified in 0.1 ml of complete Freund's adjuvant (CFA: Difco Laboratories, Detroit, MI) and injected into the hind footpads. After 10 days, the draining popliteal lymph nodes were removed and T cell proliferative assays were carried out to detect responses to each of the peptides separately as described [2; and see below].

To assay the immunogenicity of selected individual peptides, male NOD mice were immunized by injection into the hind footpads of 25 µg of peptide emulsified in incomplete Freund's adjuvant (IFA; Difco Laboratories, Detroit, MI). Draining inguinal lymph nodes were collected and pooled 10 days later. T cell proliferation assays were done in 96-U well plates, (2×10^5 cells/well) using complete DMEM media (200 µl/well) supplemented with 1% NUTRIDOMA-SP (Boehringer Mannheim) and 5–50 µg/ml of peptide or antigen in triplicate wells. The cultures were pulsed with [*methyl*-^{3}H]thymidine (1 µCi/well) in the last 12 h of 72 h culture, as described [2]. The results are shown at the optimal peptide concentration of 20 µg/ml as the stimulation index (SI): the ratio of the mean test cpm to the mean control cpm without antigen. SDs were always less than 10% of the mean CPM.

Peptide treatment

Peptides, 100 µg in PBS, were emulsified with an equal volume of IFA and injected subcutaneously into 10-week-old female NOD mice as described [7]. Control mice received an equal volume of PBS emulsified in IFA. The mice were monitored monthly for non-fasting blood glucose at 10:00 hours using the Blood Glucose Sensor (MediSense. Inc., Waltham, MA). Mice with a blood glucose greater than 11.1 mM were considered to be diabetic; this concentration of glucose was greater than 3 SD above the mean blood glucose concentration measured in non-diabetic mice [7].

Serum antibodies

Mice were bled monthly to detect antibody responses. The ELISA assay was done as previously described [2]. Briefly, flat-bottom Maxi-sorp plates (Nunc, Roskilde, Denmark) were coated with 100 µl per well of peptide in PBS, at a concentration of 10 µg/ml, for 2 h at RT followed by overnight incubation at 4°C. After incubation with peptide, the plates were washed and blocked for 2 h at 37°C with 7% BSA (Sigma) in PBS. Sera were diluted 1:50 then added for 2 h at 37°C, followed by incubation for 2 h with 100 µl per well of goat isotype-specific anti-mouse IgG (gamma chain Fc-specific) conjugated to alkaline phosphatase (Jackson, Philadelphia, PA). After washing, the plates were incubated with the substrate p-nitrophenyl/phosphate (P104; Sigma) and read using an ELISA reader at 405 nm. The results at 7 months of age are shown.

Statistics

Statistical analyses were done using the chi-square test and the Student's *t*-test where appropriate.

Results

T cell proliferative responses

Spontaneous T cell responses in prediabetic NOD mice have been detected to the p277 peptide [2, 3, 12] and to larger fragments of the mouse hsp60 molecule that contain the p277 sequence [3]. To detect other T cell epitopes on the mouse hsp60 molecules, we immunized NOD mice with pools of peptides overlapping the hsp60 sequence and found that all mice showed strong responses to p12, and lesser responses to some other hsp60 peptides; a response to p277 is shown for reference (Figure 1). To confirm the immunogenicity of p12 alone, male NOD mice were immunized with p12, p277, or other peptides immunogenic for NOD mice, the MT-p278 peptide (residues 431–447 in the mycobacterial hsp60 molecule), and GAD-p35 (residues 524–543 in the GAD65 molecule). Figure 2 shows that p12 was immunogenic, as were p277 and MT-p278; GAD-p35 was also immunogenic, but less so. Hsp60 peptide p38 was not immunogenic. None of the peptides were cross-reactive; the T cell proliferative responses were limited to the immunizing peptide (not shown).

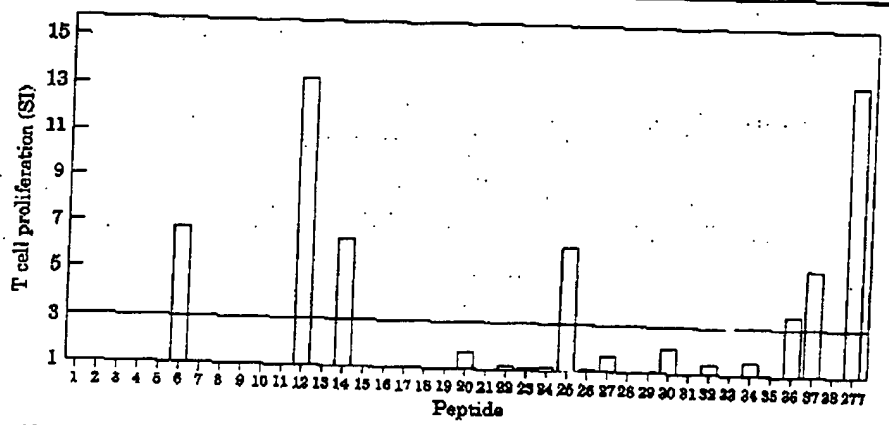

Figure 1. Detection of hsp60 peptides immunogenic for NOD mice. Groups of male NOD mice were immunized in the hind footpads with pools of four peptides in CFA containing 25 μg of each peptide. After 10 days, the draining popliteal lymph node cells were assayed *in vitro* for T cell proliferative responses to each of the peptides in the pool. Peptide p277 is included among the 38 overlapping peptides (see Table 1). The medium control cpm were 2,000 in each group. Two additional experiments produced a similar pattern of reactivities. The horizontal line at SI=3 is included as a reference for significant T cell proliferation

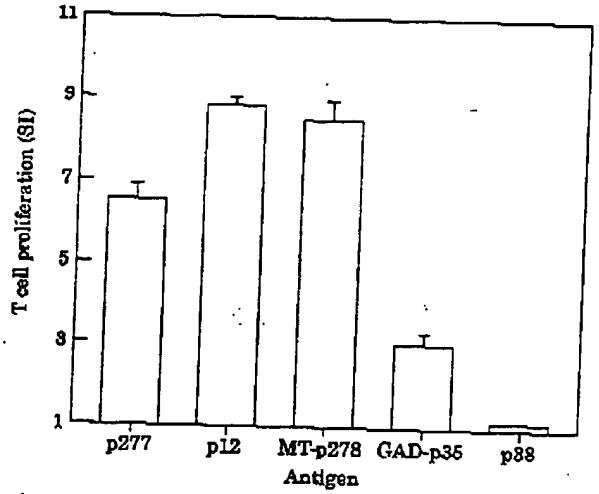

Figure 2. T cell proliferative responses induced to peptides. Groups of three to five male NOD mice were immunized with peptides p12, p277, p38, GAD-p35, or MT-p278 in IFA and the draining lymph nodes were assayed for T cell proliferative responses to the peptides. The following cpm values were obtained in the medium controls without peptide: p12, 881; p277, 1243; MT-p278, 698; and GAD-p35, 1430. The SD values are indicted by the bars.

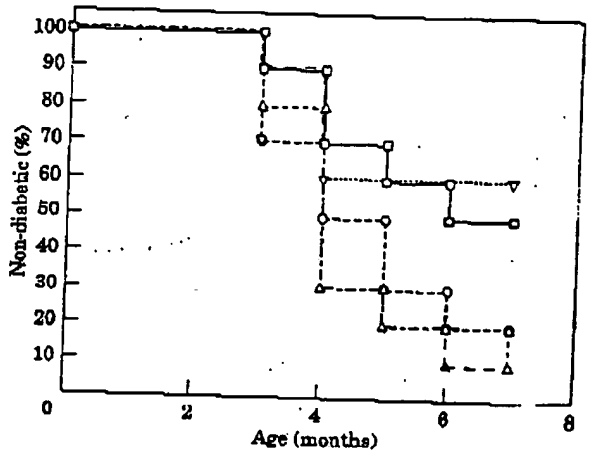

Figure 3. Effect of peptide administration on diabetes. Groups of 10–20 NOD mice were treated at 10 weeks of age with 100 μg of p12, p277, p35-GAD, or MT-p278 in IFA, or IFA alone. The mice were bled monthly and followed for the onset of hyperglycemia. As compared to the IFA-treated control group, the mice treated with p12 and p277 were significantly protected, $P<0.05$. □, p12; ▽, p277; △, MT-p278; ○, GAD-p35; ▲, IFA.

A longitudinal study of female NOD mice at age 3–16 weeks showed no spontaneous T cell proliferative responses to p12 in their spleens (not shown), although responses to p277 and to whole hsp60 were seen as described [2, 3, 12]. Thus we had in hand four immunogenic peptides: p12 and p277 from the mammalian hsp60 molecule, GAD-p35 from the diabetes-associated GAD65 molecule, and MT-p278, a foreign immunogen. Of these, spontaneous responses were detected to only p277 and MT-p278 [12].

Peptide treatment

Following a protocol shown to be effective with p277 [2, 6, 7, 12], groups of 10-week-old female NOD mice were treated by a single subcutaneous injection of each peptide (100 μg) emulsified in IFA. The mice were observed for the development of diabetes up to 7 months of age. Figure 3 shows that peptides p277 and p12 were both effective in inhibiting the development of diabetes; 60 and 50% of the mice, respectively, were free of hyperglycemia at 7 months of age ($P<0.05$). In contrast, treatment with peptides

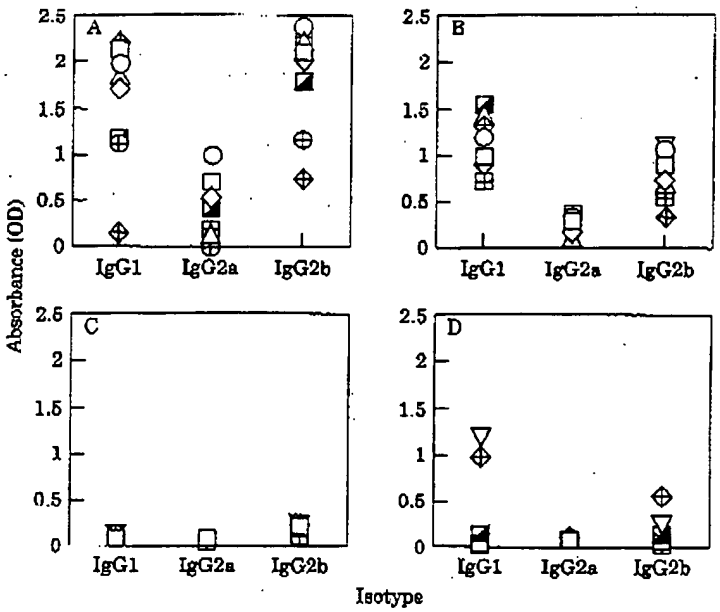

Figure 4. Antibody isotypes in response to peptide treatment. Mice, 10 per group, were treated as described in the legend to Figure 2. Individual mice were analysed monthly for antibodies to (A) p12; (B) p277; (C) GAD-p35; and (D) MT-p278, of the IgG1, IgG2a and IgG2b isotypes. The results are shown at 7 months of age. The level of significance of the prevalence of IgG1 and IgG2b antibodies in groups A and B compared to IgG2a is $P<0.001$. The differences between the levels of IgG1 and IgG2b antibodies compared to the IgG2a antibodies in groups A and B were significant ($P<0.001$).

MT-p278 or GAD-p35 was no different from treatment with IFA alone; 90% of the mice manifested hyperglycemia. Three repeated experiments showed essentially the same results.

Antibodies

Successful treatment of STZ-induced diabetes [9] or of spontaneous NOD diabetes [12] with peptide p277 was associated with the appearance of anti-peptide antibodies predominantly of the IgG1 and IgG2b subtypes. We therefore examined the peptide-treated NOD mice for their serum antibodies at 7 months of age. Figure 4 shows that the two peptides effective in arresting diabetes, p12 and p277, were also effective in inducing strong antibody titers of the IgG1 and IgG2b isotypes that were significantly greater than the IgG2a antibody titers in these groups ($P<0.001$). The lower amounts of IgG2a antibodies were not a technical artefact because we could readily detect the predominance of IgG2a antibodies to other antigens in NOD mice (see [12]). The mice treated with peptides MT-p278 or GAD-p35 did not respond as strongly; none of the GAD-p35-treated mice produced specific IgG1 antibodies and only two of the 10 MT-p278-treated mice produced antibodies of the IgG1 isotype. The mice treated with MT-p278 or GAD-p35 showed significantly lower titers of IgG1 and IgG2b antibodies ($P<0.001$). Similar results were obtained in two additional experiments. There was no cross-reactivity between any of the antibodies (not shown). Thus, effectiveness in inhibiting diabetes was associated with the induction of an antibody response mainly of the IgG1 and IgG2b isotypes.

The relationship between an effective therapeutic response and the titer of antibody was confirmed by a comparison of the concentration of blood glucose with the concentration of IgG antibodies in individual mice at 7 months of age. Figure 5 shows that mice with higher titers of IgG anti-p12 antibodies tended to have lower blood glucose concentrations; conversely, the p12-treated mice that produced little antibody to p12 tended to have high blood glucose ($P<0.004$).

Discussion

In this investigation, we screened NOD mice for their T cell proliferative responses to a set of peptides overlapping the sequence of the mouse hsp60 molecule. A number of peptides appeared to be immunogenic: p6, p12, p14, p25, p36, and p37 (Figure 1). These peptides probably do not exhaust the T cell epitopes in mouse hsp60 because, for practical reasons, the immunizations used peptide pools and competition between peptides in the pools could have obscured their immunogenicity. Indeed, p30, which was very poorly immunogenic in the pool immunization, contains most of the naturally immunogenic p277 sequence (see Tables 1 & 2). Thus, other potentially immunogenic hsp60 peptides could also have been missed in this screening. Nevertheless, we succeeded in identifying p12, which is an effective T cell immunogen. In fact, p12 is strongly bound by the NOD MHC class II molecule and served as a reference

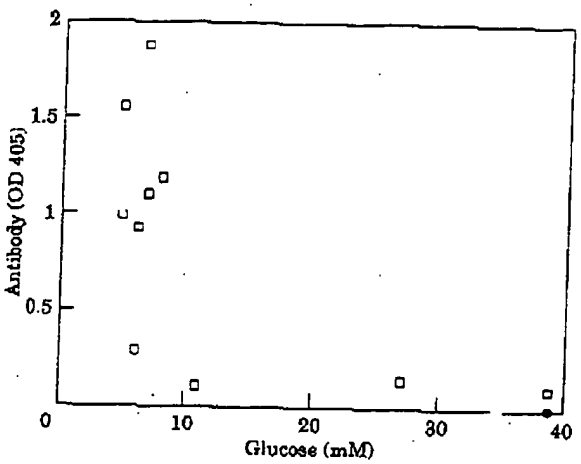

Figure 5. Inverse relationship between antibodies and blood glucose. Groups of female NOD mice were treated with p12 (10 mice) or with IFA alone (nine mice) as described in the legend to Figure 2. The amount of anti-p12 specific antibody is plotted together with the blood glucose concentration measured at 7 months of age. The degree of negative correlation between high antibodies and blood glucose was significant using the Spearman rank correlation: $r=-0.73$, $P=0.0004$. □, p12 IFA; ●, IFA.

peptide to analyse the peptide-binding motif of the IA^{g7} molecule [13]. The results presented here indicate that peptide p12 of the mouse hsp60 molecule, like peptide p277, can be effective in treating mice close to the outbreak of overt hyperglycemia. In contrast to p277, we did not observe spontaneous T cell proliferative responses to p12 in the spleens of prediabetic NOD mice. Thus, a spontaneous anti-peptide proliferative response detectable in the periphery is not a requirement for a peptide to be effective in blocking the diabetic autoimmune process. There is no way of knowing, as yet, whether anti-p12 T cells are present in the islets.

The finding that peptide p277 is not the only hsp60 peptide that can modulate NOD diabetes is significant. It was conceivable that the involvement of hsp60 in NOD diabetes could have come about by mimicry between the p277 peptide of hsp60 and some unknown molecule more specific for β-cells [3, 14]. However, the effectiveness of a second hsp60 peptide, p12, supports the conclusion that the hsp60-like molecule functional in diabetes is probably hsp60 itself [3].

The failure of peptides MT-p278 and GAD-p35 to arrest the development of diabetes indicates that not any self-antigen or spontaneously reactive T cell antigen can be used to abort the autoimmune process. It is interesting that MT-p278 failed to induce high titers of antibodies or to protect, despite the fact that this peptide binds IA^{g7} strongly [13] and is strongly immunogenic for NOD T cells (see Figure 2 and [12]). Moreover, the induction of antibodies of any specificity does not necessarily affect NOD diabetes; treatment of NOD mice with BSA, which induces high titers of antibodies as well as strong T cell responses (not shown), does not affect the development of diabetes [6]. Although GAD-p35 was not found by us to be as strongly immunogenic for T cells as were the other peptides (Figure 2), NOD mice have been reported to manifest spontaneous T cell responses to this peptide [15]. The administration of the whole GAD65 molecule in IFA was reported to arrest the disease [16]. Thus, the administration of the GAD-p35 peptide alone may not have provided an adequate therapeutic stimulus. It is interesting that treatment with p277 was found to downregulate autoimmunity to GAD65 epitopes [12], and vice versa, treatment with whole GAD65 was reported to downregulate the spontaneous T cell reactivity of NOD mice to p277 [16]. The spontaneous responses to p277 [2] and to GAD-p35 [15] may be explained by the involvement of these peptides in the autoimmune process. Why p12 differs from p277 in not eliciting spontaneous T cell reactivity *in vitro* is not known; perhaps p12 is more cryptic [17] than p277. The spontaneous responses to MT-p278 can be explained by colonization of the mice with normal mycobacterial flora that may express a similar peptide sequence.

Finally, the association of effective treatment with induction of antibody specific to the peptide suggests that the therapeutic effects of p12, like those of p277 [12], might be related to the activation of Th2-like T cells responsible for helping the induction of specific IgG1 antibodies, antibodies regulated by the production of IL-4 [18]. Such T cells could suppress the Th1 T cells thought to be responsible for damaging the β-cells [11, 12, 16, 19]. Although peptides p277 and p12 also induced peptide-specific antibodies of the IgG2a isotype, thought to be dependent on the Th1-type cytokine IFN-γ [20], the amounts of these antibodies were significantly less than the amounts of the IgG1 antibodies. Thus, the cytokine balance was weighted more on the Th2 side of the scale. The cytokine required for the induction of IgG2b antibodies appears to be TGF-β, a cytokine with known suppressive effects [20, 21]. Further studies are needed to confirm directly the involvement of particular cytokines. It remains to be seen whether the antibodies to the hsp60 peptides induced by peptide treatment can actually affect the disease process or only serve to mark the cytokine shift [12]. Be that as it may, the predominance of peptide-specific antibodies bearing the IgG1 and IgG2b isotypes appears to be an indicator of a beneficial response to the peptides.

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Prevention of diabetes in the NOD mouse: implications for therapeutic intervention in human disease

Mark A. Bowman, Edward H. Leiter and Mark A. Atkinson

The prevention of insulin-dependent diabetes (IDD) in humans remains an elusive goal, despite the broad spectrum of therapeutic interventions that prevent the development of IDD in the non-obese diabetic (NOD) mouse. Can an animal model in which spontaneous autoimmune pathology is interrupted so easily serve as an archetype for the design of clinical trials aimed at the prevention of IDD in humans? In this article, Mark Bowman, Edward Leiter and Mark Atkinson review the intervention strategies that prevent IDD in the NOD mouse and indicate why these studies may well be relevant to the prevention of IDD in humans.

Many key features of human insulin-dependent diabetes (IDD) are reflected in the non-obese diabetic (NOD) mouse: the development of insulitis, whereby pancreatic islets of Langerhans are infiltrated by lymphocytes that are selectively cytotoxic to the insulin-producing β cells; the inheritance of particular major histocompatibility complex (MHC) class II alleles, representing the major component of genetic susceptibility; the transmission of IDD by hematopoietic cells in bone marrow; and the T-cell dependence of disease pathogenesis[1–5] (Table 1). The origin, genetics and immunological characteristics of the NOD strain, as well as the ability of environmental manipulations to effect the diabetogenic processes in these mice, have recently been reviewed[3,6,7]. For reasons of brevity, the reader is referred to these reviews for references to much of the information described below.

There are relatively few characteristic differences between human and mouse disease. The presence of a high percentage of T cells (both $CD4^+$ and $CD8^+$ subsets) in NOD lymphoid tissues and peripheral blood distinguishes NOD mice from humans with IDD and from the severely T-lymphopenic diabetes-prone BB/Wor rat, which also spontaneously develops autoimmune IDD (Ref. 8). NOD mice do not display the severe diabetic ketoacidosis characteristic of untreated human patients with IDD, perhaps due to an enhanced ability of mice to metabolize blood ketones to lactate. In addition, NOD mice exhibit a pronounced female gender bias for disease susceptibility that is not observed in BB rats or in humans. While the specific reasons for this difference are unclear, female mice are known to be stronger immunological responders to exogenous stimuli than males and it has been demonstrated that the lower incidence of IDD in NOD males is partially regulated by gonadal sex steroids. Finally, the pathogenesis of IDD in NOD mice is associated with expression of endogenous defective retroviruses[9] in β cells, a feature that has not been described previously in the β cells of insulitic islets from BB rats or humans.

One major distinction must be considered when comparing IDD etiopathogenesis in humans and NOD mice. Given the genetic heterogeneity within the human population, the development of IDD is likely to reflect heterogeneous mixtures of susceptibility genes whose penetrances are responsive to different thresholds of intragenic and environmental influences. NOD mice inherit the same gender-specific set of susceptibility genes, a consequence of over 50 generations of sibling matings. The penetrance of these genes can be analysed under constant, well-defined environmental conditions of diet, temperature and exposure to pathogenic agents. Consequently, the natural history of IDD development in a well-maintained NOD colony is quite predictable. Indeed, an intervention study in NOD mice can be designed such that treatment is initiated at a presymptomatic stage prior to the occurrence of insulitis (up to 3 weeks postpartum), or before the onset of disease (four to eight weeks postpartum), at a time when considerable numbers of β cells are still intact. By contrast, the genetic and environmental heterogeneity associated with the natural history of IDD in humans is such that the age of disease onset is extremely broad and may occur at any time from the first years of life to well beyond 50 years of age. Given these complexities, it has been difficult for clinical investigators to develop simple diagnostic tools for the early identification of humans destined to develop IDD. For these reasons, studies to prevent IDD in NOD mice must be carefully analysed for their applicability to therapeutic intervention in human disease.

Table 1. Comparison of insulin-dependent diabetes in humans and NOD mice

Characteristic	Humans	NOD mice
Genetic predisposition (MHC class II linkage)	+	+
Complex polygenic control	+	+
Environmental effects on gene penetrance	Probable	+
Disease transmissible *via* bone marrow	+	+
T-lymphocyte-driven insulitic lesions	+	+
Leukocytic infiltrates found in other organs	Sometimes	+
Defective peripheral immunoregulation	+	+
Humoral reactivity to β cells	+	+
Endogenous retroviral genes expressed in β cells	−	+
Diabetic ketoacidosis if untreated	+	Mild
Gender bias	±	+
Successful intervention therapies	Ongoing	+

Role of genetics and the environment

The inheritance of susceptibility to disease in humans and NOD mice is polygenic. In NOD mice, homozygosity of the MHC class II region of the unique H-2^{g7} haplotype is necessary, but not sufficient, for development of overt IDD (Ref. 7). Destruction of sufficient numbers of β cells to produce persistent hyperglycemia and glycosuria, the clinical phenotype of IDD, requires a complex interaction with numerous other genes that are not linked to the MHC locus. Although the relatively high discordance rates for IDD in monozygotic twins suggest an important role for the environment in human IDD, an environmental influence has not been unequivocally proven[8]. By contrast, it has been clearly demonstrated that penetrance of diabetes-susceptibility genes in the NOD mouse is strongly influenced by agents in the extrinsic environment, including dietary components and microbial pathogens[10,11].

The protective effects of exposure to microbial pathogens is of considerable interest for two reasons. First, while there are epidemiological and anecdotal data suggesting that viruses may precipitate the autoimmunity that results in human IDD (Ref. 12), viral and bacterial infections have more often been reported to reduce rather than exacerbate the incidence of diabetes in the NOD mouse. These data contradict the paradigm of molecular mimicry, wherein a microbial antigen is sufficiently similar to self antigens to provoke a pathogenic cross-reactive autoimmune response. Second, although many current therapies for autoimmune disease involve immunosuppression, the effect of microbial challenge on diabetes in the NOD mouse presumably occurs through immunostimulation[7].

A recent analysis of worldwide NOD mouse colonies showed that the cumulative incidence of diabetes at 30 weeks is more variable (as well as lower) in males than in females[13]. Although some of the colony differences may be explained by genetic divergence amongst substrains of NOD mice separated from the original source colony, most of the differences appear to be environmentally driven. NOD males are particularly susceptible to modulation of diabetes development, since Caesarian transfer of pups from a conventional environment to a pathogen-free environment markedly increases the incidence of diabetes[14].

Developmental and functional defects have both been reported in the antigen-presenting cells (APCs) of NOD mice[15,16]. These defects appear to perturb presentation of self antigens in the course of tolerance induction. Some of these defects are associated with defective secretion of endogenous cytokines, including interleukin 1 (IL-1), IL-2 and IL-4 (Refs 17,18). It is possible that exposure to microbial pathogens may counterbalance these defects *via* elevation of inflammatory cytokine levels. Indeed, exposure of NOD mice to viral pathogens such as encephalomyocarditis virus (EMCV), lymphocytic choriomeningitis virus (LCMV) and murine hepatitis virus (MHV), or to bacteria (e.g. *Mycobacterium* and *Streptococcus*) and their components (e.g. complete Freund's adjuvant (CFA) and OK432), prevents and/or delays the onset of IDD (Refs 19–25). This may result from an upregulation of APC function by inflammatory cytokine release, since the IDD-protective effects produced by virological challenge to the immune system can be mimicked by treating young NOD mice with poly [I:C], a potent inducer of immune interferons[17]. The pleiotropic actions of the cytokine cascades that are initiated by treating NOD mice with viruses, bacteria and bacterial products result in the generation of an increased number of functional immunoregulatory cells (including T cells) capable of suppressing diabetogenesis[2,25].

Therapeutic interventions

Intervention strategies in the NOD mouse can alter the incidence of insulitis, spontaneous diabetes or cyclophosphamide-induced diabetes. However, it is important that the incidence of overt IDD, rather than insulitis, is used as an experimental readout in intervention studies, since insulitis is an uncertain predictor of IDD development. Hence, this review will only consider protocols that clearly alter the rate or total incidence of IDD development in colonies of NOD mice in which the investigators have demonstrated a high incidence of spontaneous or cyclophosphamide-induced diabetes in controls. Furthermore, diabetes-related studies in transgenic mice will not be discussed.

Despite limiting discussion to these stated criteria, successful interventions are remarkably abundant and diverse (Box 1). Indeed, the ease of manipulating IDD incidence in the NOD mouse attests to the utility of this model for dissecting the multiple components of a complex disease. The explanation for the success in identifying such a broad spectrum of factors capable of retarding or preventing IDD in NOD mice may be quite straightforward. Each mouse inherits the same set of diabetes-susceptibility genes and, by controlling the physical and biological environment in which these genes are expressed, investigators have established a longitudinal progression of immunological and endocrinological events leading to IDD (Refs 26,27). For example, in NOD females, pancreatic islet-infiltrating leukocytes are first observed at three to five weeks postpartum, whereas a significant decline in pancreatic insulin content is not detected until

approximately 12 weeks. The clinical presentation of hyperglycemia and glycosuria typically occurs at age 16–20 weeks in 50% of fully mature NOD females. The early infiltrates contain fewer T-cell effectors, as demonstrated by the finding that adoptive transfer of splenic T cells from young prediabetic mice into NOD–severe combined immunodeficiency (NOD–SCID) mice requires a longer period before onset of IDD than does transfer using an equal number of cells from diabetic donors[28].

Thus, a broad 'window' for therapeutic intervention exists between the time that mononuclear infiltrates first surround the pancreatic islets and the time that β-cell numbers fall below a level required to maintain normoglycemia. Therapeutic protocols that have been used successfully to interrupt the pathogenic process in NOD mice (summarized in Box 1) can be grouped into the following categories: (1) immunosuppression; (2) immunostimulation; (3) tolerance induction or placing β cells in a metabolic 'resting' state; (4) manipulation of the hormonal or dietary milieu; and (5) treatment with anti-inflammatory agents.

Immunosuppression

T-cell deficient NOD mice homozygous for either the nude (nu) or SCID mutation do not develop insulitis or diabetes. Furthermore, treatments that compromise the effector functions or viability of either the $CD4^+$ or $CD8^+$ T-cell subsets can retard or circumvent diabetes. Immunosuppressive agents capable of retarding or preventing IDD onset include cyclosporin A, FK506, rapamycin and deoxyspergualin[29–31]. Similarly, successful intervention studies employing an assortment of antibodies directed towards the T-cell surface also exist, including antibodies to the T-cell receptor (TCR) α and β chains, anti-lymphocyte serum, anti-CD3, anti-Thy 1.2, anti-CD4 and anti-CD8 (Refs 31–34). It appears that B lymphocytes are not required at the effector level, since adoptive transfer of NOD T lymphocytes into anti-μ suppressed neonatal recipients still results in diabetes. NOD mice do not express lytic complement activity due to a mutation in the *Hc* gene encoding C5a (Ref. 35).

It has been found that 'irrelevant' control monoclonal antibodies to certain TCR Vβ clonotypes that are not present in NOD mice unexpectedly reduce the incidence of disease. However, this protection may be mediated by nonspecific immunostimulation as a consequence of an antiglobulin immune response. Indeed, it is not uncommon for NOD mice to produce antibodies against the monoclonal antibodies being tested for therapeutic potency.

Immunostimulation

A striking characteristic both of NOD mice and patients with IDD is a markedly depressed autologous mixed lymphocyte reaction (AMLR), which in turn suggests a defect in peripheral immunoregulatory mechanisms. Defects in cytokine-elicited differentiation and maturation of APCs from the bone marrow of NOD mice may result in the inefficient presentation of self antigens and impair the tolerogenic capacity of these cells. These defects could explain why exposure of prediabetic NOD mice to a number of environmental pathogens imparts resistance to diabetes. Thus, certain environmental stimuli may upregulate APC function and (1) increase thymic deletion (or thymic or peripheral anergization) of autoreactive T-cell clones, or (2) potentiate the activation of immunoregulatory T lymphocytes in the periphery, or tolerize by a combination of these mechanisms. This concept is reinforced by the finding that chronic administration of a variety of cytokines, or single injections of potent immunomodulators that upregulate endogenous cytokine expression, also circumvent diabetes (Box 1). Indeed, many therapeutic manipulations may stimulate antigen processing and presentation by macrophages. Protection associated with some of these treatments, for example administration of IL-1 or IL-2, restores a more-normal AMLR, although chronic treatment with IL-4 does not.

Box 1. Therapies that prevent diabetes in the NOD mouse

Immunosuppression
T-cell functions: neonatal thymectomy, anti-lymphocyte serum, anti-Thy-1, anti-CD3, anti-CD4, anti-CD8, cyclosporin, FK-506.
Macrophage/APC functions: anti-complement receptor, silica, LDHV, anti-MHC class I, anti-MHC class II, blocking peptide for MHC class II, anti-IFN-γ.

Immunostimulation
Pathogenic viruses: LCMV, EMCV, MHV.
Cytokines or cytokine inducers: IL-1, TNF-α, IL-4, IFN-γ, poly [I:C], Con A.
CFA, BCG, OK432, heat-shock protein 65.

Tolerance induction
Bone-marrow transplantation.
Intrathymic islet transplantation.
Oral insulin.
Dendritic cells from pancreatic node.
Neonatal tolbutamide treatment.
Immunization with insulin or insulin B chain.

Manipulation of hormonal/dietary milieu
Gonadectomy.
Prophylactic insulin treatment.
Diazoxide.
Elevated temperature.
Semi-purified diets.

Anti-inflammatory agents
Nicotinamide, superoxide dismutase-desferrioxamine, vitamin E, aminoguanidine.

Abbreviations: APC, antigen-presenting cell; LDHV, lactate dehydrogenase virus; MHC, major histocompatibility complex; IFN-γ, interferon γ; LCMV, lymphocytic choriomeningitis virus; EMCV, encephalomyocarditis virus; MHV, murine hepatitis virus; IL, interleukin; TNF-α, tumor necrosis factor α; Con A, concanavalin A; CFA, complete Freund's adjuvant; BCG, Bacille Calmette Guérin.

Other immunological defects characteristic of NOD mice may also be ameliorated by immunostimulation. These include: subnormal lipopolysaccharide (LPS)-stimulated IL-1 secretion; subnormal secretion of IL-2 and IL-4 by splenic and thymic T cells, respectively; and depressed thymocyte responses to mitogenic stimulation.

Tolerance induction or β-cell 'rest'

A single injection of anti-CD3 monoclonal antibody into neonatal NOD mice is tolerogenic[36]. Tolerance has also been achieved through the selective destruction or self-inactivation of autoreactive T cells, without damaging the function of all T cells. Thymic deletion of autoreactive clones can be enhanced by intrathymic injection of islet cells into neonatal or adolescent NOD mice[37]. Similarly, the peripheral deletion of autoreactive clones has been achieved *via* vaccination with autoreactive T-lymphocyte lines[38], activated $CD4^+V\beta8^+$ T cells[39], and T-cell clones specific for a 65 kDa heat-shock protein[40]. Other protocols, such as the oral administration of insulin[41], or the injection of dendritic cells or splenocytes[42], may be more likely to induce regulatory tolerance or clonal anergy in T lymphocytes reactive to islet antigens.

Prophylactic insulin therapy prevents diabetes and the formation of insulitis in NOD mice[43]. Whether or not this mechanism is due to β-cell 'rest' (Ref. 44 and see below), or to the induction of tolerance, is unknown. According to the concept of β-cell rest, chronic treatment with diazoxide suppresses insulin secretion and reduces diabetes by causing the β cells to be less visible to the immune surveillance system, or less susceptible to inflammatory damage. However, a recent study demonstrated that a potent insulin-stimulator, tolbutamide, also reduced the incidence of IDD (Ref. 45). Furthermore, administration of insulin, either orally or intraperitoneally (i.p.), protects from diabetes and i.p. injections of the insulin B chain is as protective as intact insulin[46]. The similar effect of oral and i.p. administration of whole insulin argues that prophylactic insulin therapy protects *via* insulin tolerization, rather than through β-cell rest. Indeed, recent studies indicate that the IDD-protective effects of intrathymic or intravenous administration of recombinant glutamic acid decarboxylase (GAD-65) into NOD females during weaning are achieved by acquisition of T-cell tolerance to this candidate β-cell autoantigen[47,48]. Although stimulation in early life may not be applicable to the period of β-cell destruction, the concept of β-cell rest has also been questioned by the observation that neonatal glucose treatment in NOD mice reduces diabetes frequency and stimulates insulin secretion[49].

Treatments that may interfere with the presentation of antigenic peptides to T cells include the silica-mediated destruction of macrophages[50], as well as the administration of antibodies against mouse MHC class II I-A molecules[51], and 'blocking' peptides[52] that compete for binding to $I\text{-}A^{g7}$. However, antibody blocking of macrophage complement receptors probably protects by preventing the recruitment of these cells into insulitic lesions. The protection against diabetes that results from infection with lactate dehydrogenase virus (LDHV) may also be mediated *via* effects on the macrophage population.

Manipulation of hormonal/dietary milieu

It has been found that the occurrence of diabetes in mouse colonies with a low incidence of spontaneous diabetes in males increases after castration and that androgen treatment protects females[53,54]. The protective effect of semi-purified diets may be mediated by the alteration of cytochrome p450 activities in the liver which, in turn, may control the level of active androgens and estrogens in tissues (E.H. Leiter *et al.*, unpublished). The reduced incidence of diabetes that is observed when temperatures are increased may relate to a decreased intake of diabetogenic natural-ingredient diets. Although bovine milk proteins have sometimes been associated with the diabetogenic properties of natural-ingredient chow diets, the association was not reproduced in NOD colonies with a high incidence of spontaneous diabetes.

Anti-inflammatory agents

One strategy for preventing diabetes in NOD mice is based on the assumption that destruction of β cells results from the release of oxygen radicals by macrophages. Administration of oral nicotinamide, superoxide dismutase-desferrioxamine and vitamin E have the effect of reducing such oxidative processes in β cells[55,56]. Other preventative therapies arise from the suggestion that IL-1 and other inflammatory cytokines induce islet β cells to produce toxic levels of nitric oxide, resulting in cellular suicide. A recent report claiming prevention/delay in the adoptive-transfer model of IDD using aminoguanidine (a competitive inhibitor of nitric oxide synthase) supports a role for nitric oxide production in the pathogenesis of β-cell destruction[57].

Human trials for IDD prevention

To date, the major research effort directed at prevention of IDD in humans has focused on the effects of nonspecific immunotherapy administered at the time of diagnosis of IDD, such as cyclosporin and azathioprin. Pharmacological agents such as these have had an extensive history of use in a variety of clinical situations, for instance in organ transplantation and rheumatoid arthritis. Multiple investigations have demonstrated that treatment of new-onset IDD patients with these agents leads to the ability of some patients to produce insulin (i.e. C-peptide) over prolonged periods, and a number of anecdotal reports have described patients who have undergone clinically significant remissions of disease[58,59]. Unfortunately, in most individuals, the effect of these drugs was not lasting enough to be of clinical significance and reports of permanent remissions from IDD were rare.

Human IDD is preceded by a long presymptomatic period – suggesting that the clinical symptoms of disease arise only after the destruction of the majority of β cells. Many researchers believe that effective methods of disease prevention will require therapy at an earlier, preclinical asymptomatic stage. This preclinical period

can be identified effectively through autoantibody markers of anti-islet immunity, with a majority of studies to date utilizing the islet-cell cytoplasmic autoantibody[1,2]. However, the use of immunosuppressive agents in asymptomatic individuals is controversial. Problems associated with the long-term use of immunosuppressive agents include toxic side-effects, such as nephrocytotoxicity, and the increased frequency of developing viral infections and cancer (e.g. B-cell lymphomas)[60]. These risks have led to a search for less-aggressive interventions that prevent IDD without impairing normal immune functions. Although most of this research has involved studies of diabetes interventions in the NOD mouse, a number of these studies are now being considered for human clinical trials. Prophylactic insulin therapy prevents diabetes in each of the rodent models for IDD (BB/Wor rats and NOD mice) and, in NOD mice, the therapy also prevents insulitis[41]. Prophylactic insulin has recently undergone pilot trials in humans and shows promising preliminary results[61].

Anti-inflammatory agent inhibitors have also shown promise in preventing IDD in NOD mice. For this reason, oral nicotinamide has been used in two pilot studies to prevent IDD in asymptomatic individuals who have increased risk for IDD, and preliminary results indicate success[62]. The beneficial effects of this drug may derive from prevention of β-cell damage by oxygen or nitrogen (e.g. nitric oxide) free radicals. However, although nicotinamide is commonly viewed as a safe drug, higher doses than those used in human clinical trials have reportedly resulted in liver dysfunction[63].

Another interesting strategy that is being considered for clinical trials in humans is the oral induction of tolerance to islet-cell proteins that have been implicated as autoantigens in anti-islet-cell immunity. Indeed, the onset of diabetes was both attenuated and protracted in NOD mice fed with porcine insulin[41]. Although the mechanism for this effect is unresolved, it may be mediated by the generation of regulatory T cells that are reactive to insulin. After migrating to the pancreatic islets and encountering insulin, these lymphocytes may secrete regulatory cytokines such as IL-10 and transforming growth factor β (TGF-β), and thereby suppress bystander autoimmune responses. Recent studies in NOD mice have shown that parenteral administration of insulin is as effective as oral administration in preventing IDD (Ref. 46). Given the paucity of side effects, as well as the targeted nature of these antigen-based therapeutic approaches, the oral and/or parenteral administration of either GAD (Refs 47,48) or insulin may have great applicability to the prevention of IDD in humans.

The prevention of IDD in rodents can also be achieved by immune-enhancement therapy. This approach, which at first appears paradoxical, is based on the hypothesis that the essential problem underlying IDD is the inability to maintain peripheral immunological tolerance to the pancreatic islets actively. Thus, any number of nonspecific immunization events may enhance general tolerogenic mechanisms sufficiently so as to prevent disease. Indeed, the administration of carefully timed doses of CFA has been shown to prevent diabetes in NOD mice[64]. Similarly, treatments with recombinant IL-1, IL-2, tumor necrosis factor α (TNF-α) and poly [I:C] can all prevent diabetes[65]. Human trials that will test this hypothesis by actively immunizing individuals with Bacille Calmette Guérin (BCG) are planned.

Finally, considerable interest has been generated by the observation that intrathymic transplantation of islet cells at birth in rodent models of IDD both prevents insulitis and diabetes[37,48,66]. T-cell tolerance is mediated, in part, by interactions between maturing thymocytes and self antigens presented by thymic stromal cells. The beneficial effect of intrathymic transplantation may be the result of the specific modulation of diabetogenic T cells that are forced to mature in a thymic microenvironment enriched for islet antigen. Once the autoantigen(s) that elicits human IDD is identified, autoantigen-immunization in early life may result in tolerance to the antigen and prevention of IDD. Thus, a method for the induction of specific tolerance to islet-cell antigen in humans would appear to be promising.

Conclusions

The NOD mouse has provided a model system to study not only the pathogenesis and natural history of a disease that is similar to human IDD, but also a means with which to test intervention protocols that could be used to prevent the disease in humans. Indeed, studies in NOD mice also indicate that immunostimulation may be a more viable means of keeping the prediabetic patient non-diabetic than immunosuppression.

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TYPE-I DIABETES: A Chronic Autoimmune Disease of Human, Mouse, and Rat

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INTRODUCTION

During the past two decades enough information has accumulated to classify Type-I diabetes (also termed insulin-dependent diabetes and formerly juvenile onset diabetes mellitus) as a chronic autoimmune disease. In this disorder, cells producing insulin (β cells) within the pancreatic islets are destroyed. These cells comprise the majority of islet cells but less than 2% of the total pancreatic mass (1, 2). Other endocrine islet cells such as those that synthesize the hormones glucagon or somatostatin are not destroyed in Type-I diabetes. With destruction of β cells and the resulting insulin deficiency, acute metabolic abnormalities develop which in the absence of insulin therapy lead to death in a state resembling accelerated starvation. Since 1922 and the advent of insulin therapy, the majority of acute deaths due to insulin deficiency have been prevented. Nevertheless patients with Type-I diabetes are at constant risk for severe hypoglycemia due to pharmacologic administration of insulin, and the majority develop a series of morbid and often mortal complications including renal failure, proliferative retinopathy leading to blindness, and neuropathic syndromes.

There is extensive circumstantial evidence that the incidence and severity of diabetic secondary complications correlate with the degree of metabolic control. This circumstantial evidence has been strengthened during the past decade with the availability of the means to monitor average blood

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glucose control by assays determining the amount of glycosylated hemoglobin. The pathogenic connection between the degree of metabolic control and secondary complications has not been definitively characterized but includes the loss of specialized cells such as the pericyte of retinal capillaries. In addition rare disorders associated with Type-I diabetes have recently been associated with immune processes, including stiff-man syndrome where antibodies to central GABA-ergic neurons as well as islets are frequently present (3). A diabetes-associated dermatologic condition termed necrobiosis lipoidica results from vasculitic dermal lesions and occurs in approximately 1% of patients with Type-I diabetes and has been observed to both precede and follow the development of diabetes. Finally, a series of recent studies suggest that many Type-I diabetics and non-diabetic relatives of patients with Type-I diabetics who are islet cell antibody positive have antineuronal and antiadrenal medullary antibodies (4, 5). In addition, adrenal medullary fibrosis associated with Type-I diabetes has been described (6). These studies suggesting a role of the immune system in diabetes-related complications are beyond the scope of this review. In this review we concentrate on the pathogenesis of Type-I diabetes in human, the BB rat, and the NOD mouse. We address in sequence a series of questions concerning the autoimmune and genetic processes that lead to Type-I diabetes:

A. What are the genes creating susceptibility, and on which cell type do they act?
B. What triggers the development of active autoimmunity?
C. What are the target antigens, and do any of the putative target antigens also function as an "immunogen" responsible for the initiation of autoimmunity?
D. What are the effector molecules leading to β-cell destruction?
E. What is the natural history of Type-I diabetes, and is it possible currently to predict the disease?
F. Can Type-I diabetes be prevented?

WHAT ARE THE GENES CREATING SUSCEPTIBILITY?

BB Rat

Approximately 60% of BB rats develop Type-I diabetes (7). Characteristically diabetes occurs between 60 and 120 days of age and is associated with massive lymphocytic islet infiltrates (8). Equal numbers of male and female animals develop diabetes, and the majority of animals which do not develop overt diabetes have evidence of islet lymphocytic infiltrates

and β-cell destruction (8). Bone marrow cells appear to determine diabetes susceptibility (9). In addition, thymus transplantation has been utilized to prevent diabetes (10). The BB rat is unique. In addition to Type-I diabetes, these animals have a severe T-cell lymphopenia, best characterized by an essentially total absence of circulating RT6 positive lymphocytes (11–13). The RT6 antigen is a T-cell differentiation molecule expressed after thymic maturation of T cells (14). This major loss of T cells is also reflected by what appears to be a total absence of CD8-positive cytotoxic cells (this point is somewhat controversial, but the CD8 positive cells of the BB rat appear to be natural killer cells and not T cells) (15, 16) and more than 90% depression in circulating CD4 and OX19 expressing lymphocytes. The lymphopenia is inherited in an autosomal recessive pattern (17, 18). This absence of RT6 positive cells is not linked to the gene coding for the RT6 antigen (12).

A number of investigators have correlated the inheritance of phenotypic markers such as the BB's severe T lymphopenia and genetic markers for the major histocompatibility complex with the development of diabetes (17–22). These studies generally indicate that F1 animals do not develop diabetes and are not lymphopenic. Approximately 2% of F2 animals in crosses with a series of different normal rat strains (which are not RT1-U class II) develop overt diabetes. Development of diabetes correlates with inheritance of both the severe lymphopenia and BB rat's MHC (17, 18, 23). Development of diabetes in non-lymphopenic BB-derived rats has been described (18, 24), but in the BB/W strain diabetes is an extremely rare event such that no permanent nonlymphopenic strain has been created. In one cross of a substrain of BB rats with very high diabetes incidence (90%) with a control nonlymphopenic BB strain, diabetes in the F2 generation again correlated with lymphopenia, but approximately 25% of the diabetic animals were nonlymphopenic (18). Development of a nonlymphopenic diabetic strain has not been reported from these breeding studies. The authors interpreted the presence of nonlymphopenic diabetic animals as recombination between a "diabetes" gene and a lymphopenia gene. We believe it is more likely that they observed the development of diabetes secondary to a combination of genes substituting for the lymphopenia gene similar to the rare diabetic rats in the nonlymphopenic BB/W control strain in which diabetes onset occurs at an earlier age. If the linkage between lymphopenia and diabetes were as weak as reported for this breeding study, other crosses in which there is no dissociation between lymphopenia and diabetes (25) would be unlikely and it should be possible to create a nonlymphopenic diabetes-prone strain. In a recent similar study Butler and coworkers crossed BB rats with BB/W rats and concluded that the lymphopenia gene dramatically increases the development of diabetes;

rare diabetic nonlymphopenic rats with an early age of diabetes onset represent, they think, an alternative genetic syndrome (21, 26). Thus it is likely that the severe lymphopenic phenotype of the BB rat, inherited in an autosomal recessive fashion, increases the probability of diabetes (greater than 100 fold) but is not essential for the development of diabetes, given alternative genetic combinations. Rossini and coworkers have made the interesting observation that in the non-lymphopenic BB rat control strain (BB/W), diabetes can be rapidly induced with the injection of monoclonal antibody RT6 and depletion of RT6 positive lymphocytes (27).

In addition to the phenotype of severe lymphopenia, a series of breeding studies indicate that the development of diabetes of BB rats is strongly linked to a gene within the major histocompatibility complex (17, 22, 23, 28). Colle and coworkers have utilized MHC recombinants and diabetes susceptibility maps to the class-II region of the BB rat (29). It appears that all RT1 haplotypes which express class-II genes which are U are diabetogenic independent of class-I alleles. To date these studies in the BB rat are the strongest evidence that a class-II gene is essential for diabetes susceptibility. It is noteworthy, for our later discussion of human and NOD mouse class-II genes associated with diabetes, that Lewis rats whose class-II region is not diabetogenic, and BB rats whose class-II is diabetogenic, both lack aspartic acid at position 57 of their "DQ β" chain. In the crosses studied to date, animals homozygous for class-II RT1-U have the higher risk of developing diabetes, when they are compared to heterozygotes. T-cell receptor polymorphisms have not been linked to diabetes of the BB rat but Colle and coworkers have found a linkage of diabetes with acinar pancreatic lymphocytic infiltrates (23).

NOD Mouse

As for the BB rat, it is clear for the NOD mouse that more than one gene contributes to the development of diabetes (30–32). In particular one gene linked to the major histocompatibility complex is essential for diabetes susceptibility, but this gene by itself is not sufficient for the development of disease (33). Insulitis with relatively severe β cell loss can occur in mice lacking the MHC of the NOD mouse. NOD mice develop diabetes after 13 weeks of age, and for most colonies there is a marked excess of diabetes among female (70–80% at 30 weeks of age) in contrast with male NOD mice (below 20%) (34). The NOD mouse was derived from selective inbreeding of an ICR mouse strain in which cataracts were observed, and initially two lines were begun, one termed NON, which had mild hyperglycemia, and the NOD, which had normal glucoses. At the fifth

generation of inbreeding, severe hyperglycemia appeared in the NOD line and the NOD (non-obese diabetic) strain was derived (35).

In crosses of NOD mice with any other mouse strain, F1 mice do not develop diabetes. Approximately 3% of F2 mice and, depending on the strain combination, 12% of backcross mice develop diabetes. In crosses with normal mouse strains (C3H, BALB/cJ, NON, C57bl/6J, etc) development of diabetes is linked to the major histocompatibility complex (31–33, 36, 37). For most strain combinations, only animals homozygous for the H-2 of the NOD mouse develop diabetes. Wicker and coworkers in their crosses involving I-E negative strains have infrequently observed H-2 heterozygous mice developing diabetes. As no diabetic intra-H-2 recombinants have been bred or reported in crosses with NOD mice, the specific gene within the MHC creating diabetes susceptibility is unknown (32). McDevitt and coworkers have sequenced the class-II molecules of the NOD and find that the I-A alpha chain is identical to BALB/c (a strain with a nondiabetogenic H-2), while the I-A β chain differs from all other sequenced mouse strains by lacking aspartic acid at position 57 (38, 39). Ikegami and coworkers have sequenced I-A β of strains related to the NOD mouse, in particular the CTS and ICR mice, and have identified natural recombinants (40). Analysis of these class-II/class-I recombinants in breeding studies should allow for direct testing whether class-II molecules underlie diabetes susceptibility.

The genes of the NOD mouse have been studied by direct manipulation. An I-E transgene when introduced into NOD mice prevents insulitis (41). Breeding of I-E expressing mice such as BALB/c with NOD mice has not resulted in such a marked suppression of insulitis, however, and it is therefore unknown whether the transgene is having a unique effect. Lipes and coworkers have backcrossed a rearranged Vβ T-cell-receptor gene from a hybridoma reacting with ovalbumin onto the NOD mouse strain. The transgene by allelic exclusion suppresses utilization of the bulk of Vβ repertoire, but lymphocytes expressing this introduced Vβ transgene are capable of mediating insulitis (42).

We currently hypothesize that both the unique I-A β of the NOD and its lack of I-E contribute to diabetes susceptibility, but this hypothesis has not been tested with the breeding of recombinants and with current knowledge any gene or genes tightly linked to the NOD's H-2 may underlie the MHC's contribution to diabetes susceptibility.

Though heterozygosity at H-2 in breeding studies of NOD mice prevents overt diabetes, it does not prevent the development of insulitis. In particular Ikegami and coworkers have found marked insulitis and islet loss in backcross animals heterozygous for H-2 (37). Their studies suggest that a single autosomal recessive gene may determine the occurrence of insulitis

and that this gene is not linked to H-2. Studies by Prochazka and coworkers (31) and Ikegami and coworkers (37, 43) have suggested linkage of a diabetic gene with the theta locus on chromosome 9, but to date the use of probes surrounding theta have not been reported to improve the linkage detected. Breeding studies of Prochazka and coworkers provide evidence for a third gene in addition to the two discussed in creating diabetes susceptibility (31). It thus appears likely that a series of alleles at different loci, each necessary but not sufficient for the development of diabetes, will underlie susceptibility to diabetes. Until the specific genes associated with diabetes susceptibility and their allelic variants are identified, the pathogenesis to Type-I diabetes in the NOD mouse likely will not be understood.

Human

To date, the only linkage group clearly associated with the development of Type-I diabetes in humans is the genes linked to the major histocompatibiliy complex. Both population studies and studies of families indicate an essential contribution to susceptibility of genes within the MHC. The existence of multiple haplotypes, with alleles at diverse loci in linkage disequilibrium, make the pinpointing of diabetogenic MHC alleles difficult; in a way similar to that with the NOD mouse, the exact loci conferring susceptibility at this time cannot be pinpointed.

Disease susceptibility is not caused by mutant class-II alleles specific to Type-I diabetes. Todd and colleagues have cloned and sequenced the gene segments encoding the first domains of DRβ, DQα, DQβ in IDDM patients and in healthy controls. All the nucleotides sequences present in IDDM patients were also found in normal controls (39).

Among humans with Type-I diabetes, 95% of Caucasian individuals express either HLA DR3 and/or DR4, compared to 40% of the general population (44). HLA-DR3 and/or DR4 are associated with increased risk to develop the disease, while haplotypes with HLA-DR2 usually protect against Type-I diabetes. It has been suggested that selected HLA-DQ sequences, frequently reported as associated with DR4, are involved in the susceptibility to IDDM (45–49). Several groups have investigated differences among different HLA-DR4 positive haplotypes and they found differences of DNA restriction fragments of the genes encoding HLA class-II DQβ antigens (47, 48). Using restriction fragment length polymorphisms to analyze specific genotypic markers associated with HLA-DR4, Nepom and colleagues have identified a specific DQ β variant highly associated with Type-I diabetes (DQ3.2) (50). More than 90% of the DR4+ IDDM patients express this DQ3.2 allele. However, they found this variant in DR4+ sibling of IDDM studied whether or not they express clinical

diabetes. Such a sequence within a given family could thus be "necessary" but not sufficient for the development of diabetes.

The antigens encoded by this DQ allele can also be distinguished by monoclonal antibodies and alloantisera. A good correlation has been found between some serologically defined alleles coded by DQ β gene and susceptibility and resistance to disease (46). Epitope TA10, recognized by mAB 9w790 (45, 51), is not expressed by DQ3.2 alleles, in contrast to the nondiabetogenic HLA-DR4 related DQ3.1. Both DQ3.2 and DQ3.1 alleles differ by six amino acids, four of which are in the amino-terminal domain, potentially important for immune recognition (52). The differential IDDM susceptibility and the TA10 activity involve at least one or more of these residues. Expression studies show that mutagenesis in DQ3.2 β clones, using retroviral vectors, defines the amino acid 45 as critical for the generation of serologic epitopes characteristic of DQ3.1 β and DQ3.2 β molecules (52).

Recently Todd and coworkers suggested that position 57 of the HLA-DQ β chain may be a major determinant of susceptibility and resistance to the diabetes. They reported that the lack of aspartic acid at position 57 is strongly associated with the disease (39). The same group found that 96% of diabetic patients were homozygous non-Asp/non-Asp, compared to only 19% of the healthy unrelated controls, giving a relative risk, of 107 for non-Asp 57 homozygous individuals (53).

Sterkers and colleagues compared different haplotypes associated with HLA-DR2 and they suggest that, in HLA-DRw16 (HLA-DR2 subdivision positively associated with the disease) positive haplotypes, the amino acid 57 on the DQ β chain could play an important role in the T cell antigen presentation. They also reported that HLA-DQ molecules (DQw1) of HLA-DRw15 (a HLA-DR2 specificity previously called "DR2 long" and associated with resistance to IDDM) are different from HLA-DRw15 positive controls, suggesting that the HLA DQ gene would be involved in decreasing diabetes susceptibility (54).

Though there is data implicating residue 57 in DR4 and DR2 positive haplotypes of Caucasoids, there are important exceptions to the simple hypothesis that residue 57 is critical in disease susceptibility. Neutral residues at position 57 (alanine, serine, valine) do not confer high susceptibility to HLA-DR7 haplotypes, and some haplotypes containing aspartic acid in position 57 (DRw9) are associated with disease in Japanese populations (55, 56).

Sheehy and coworkers have recently reported data not supporting an exclusive effect of HLA DQ for diabetes susceptibility (57). They found that two DR4 subtypes (Dw4 and Dw10) were significantly associated with Type-I diabetes, with a relative risk (RR) of 6.2 and 7.0 respectively. IDDM is approximately equally associated with alleles of the DRB1 locus

(Dw4 and Dw10, combined relative risk, RR = 6.4) and the DQB1 locus (DQ3.2, RR = 5.9), and there also is a significant interaction between these DR and DQ alleles in IDDM, so that the only IDDM-associated haplotypes were those carrying the IDDM-associated alleles at both DR and DQ loci (RR = 12). Heterozygotes DR3/(DQ3.2, Dw10) or DR3/(DQ3.2, Dw4) had an absolute risk of 1 in 15; in contrast, Dw4 or Dw10 without DQ3.2, or DQ3.1 without Dw4 or Dw10, had a relative risk less than one.

Approximately 10% of individuals developing Type-I diabetes develop the disorder in association with other organ-specific autoimmune disorders such as Addison's disease, thyroiditis, Graves' disease, myasthenia gravis, and pernicious anemia (58–61). Two distinctive genetic syndromes have been recognized. The first termed polyendocrine autoimmune syndrome Type I is characterized by Addison's disease, hypoparathyroidism, and mucocutaneous candidiasis, with approximately 5% of patients developing Type-I diabetes. It is noteworthy that this syndrome has no HLA association (61), in contrast to a polyendocrine autoimmune syndrome Type II with Addison's disease, diabetes (50% of patients), autoimmune thyroid disease, etc., where the disease is highly HLA DR3/4 associated.

On Which Cell Type Do Diabetogenic Genes Act?

In both animal models of Type-I diabetes, evidence is accumulating that the genes creating diabetes susceptibility act at the level of bone marrow precursor cells (9, 62, 63). These are important findings because they imply that polymorphic genes acting at the level of pancreatic islet cells may have no role, or may play a minor role, in the generation of autoimmunity. In particular, bone marrow cells from NOD mice can transfer diabetes susceptibility to F1 mice which do not develop diabetes and even to normal mouse strains. In addition, normal bone marrow when transplanted into NOD mice prevents diabetes. Similar findings have been presented for the BB rat, though it has been questioned whether mature T cells within the marrow contribute to diabetes susceptibility. The primacy of bone marrow–derived cells in generating anti-islet autoimmunity is also indicated by the ability of islets from most but not all strain combinations to be destroyed or at least to induce insulitis when transplanted into BB or NOD hosts (64–67).

Lessons From Transgenic Mice

A series of transgenic mice has been created in order to elucidate the site of action and role of genes in the development of anti-islet autoimmunity. In particular a series of transgenics with class-I or class-II molecules driven by the rat insulin promoter have been created (68). In such transgenic

mice, β cells are induced to produce histocompatibility antigens. For most but not all of these constructs, β cells are destroyed. Even with β-cell destruction there is no insulitis, and transplanted islets are not destroyed. Death of β cells occurs even in SCID mice bearing the transgene. Thus it appears that induction of class-I or class-II molecules on β cells does not induce autoimmunity, though selected transgenes coupled to the insulin promoter can cause β-cell death. One transgenic strain expressing I-A^k develops neither diabetes nor insulitis (69). These studies severely restrict the manner in which class-II expression by β cells may contribute to the development of anti-islet autoimmunity.

Sarvetnick and coworkers have induced autoimmunity directed against islets in a transgenic mouse utilizing the rat insulin promoter to induce β cells to produce gamma interferon (70). These mice will destroy transplanted normal islet cells and show intense insulitis. Thus, this latter transgenic mouse strain indicates that it is possible to induce anti-islet immunity by targeting β cells, while the former experiments indicate that expression of class-II or enhanced class-I expression does not lead to autoimmunity.

In the BB rat current evidence indicates that class-II molecule expression by β cells is a late event in islet destruction, and controversy exists as to whether β cells of NOD mice ever express detectable class-II molecules. In both animals, however, class-I islet expression is enhanced.

Summary of Genetics

Studies of the two animal models of Type-I diabetes point to a series of genes, each necessary but not sufficient for the development of anti-islet autoimmunity. This is a particularly attractive observation in that it suggests that relatively simple mendelian inheritance of susceptibility can underlie the complex genetics of the development of diabetes. In humans, only genes linked to the major histocompatibiliy complex are clearly associated with disease susceptibility. As with both animal strains, it is essential to determine which specific histocompatibility genes and, if they exist, non-MHC genes contribute to susceptibility. It is likely (as already "proven" for the BB rat) that class-II molecules will contribute to susceptibility.

WHAT TRIGGERS THE DEVELOPMENT OF ACTIVE AUTOIMMUNITY?

The NOD mouse and BB rat are both now "inbred", yet these strains are not 100% concordant for the development of diabetes. Approximately 50% of identical twins of a Type-I diabetic do not themselves become

diabetic. Similar observations have been made for other autoimmune diseases and putative autoimmune diseases (e.g. multiple sclerosis). This implies that environmental factors may be essential for the activation of autoimmunity, and exposure to a specific environmental factor may determine which twin of a pair of twins develops diabetes.

It is clear from the animal models of diabetes and from observations in humans that environmental factors can change the probability for diabetes to develop or can trigger the development of autoimmunity. Major geographic and ethnic differences in incidence of Type-I diabetes have been reported (71, 72). Some studies suggest that disease incidence appears to be related to environmental temperatures, and that there is a greater seasonal variation in cooler climates (71). Regional variation, however, cannot easily be separated from racial variation. Caucasians have the highest incidence of diabetes in the United States, as compared to Blacks and Hispanics (73). Whether global geographic differences are due to environmental agents (virus, diet) or are secondary to genetic determinants is currently unknown. In particular without precise identification of all susceptibility alleles (HLA or not HLA associated) the influence of genetic differences cannot be estimated.

The search for environmental factors contributing to the development of diabetes in humans—perhaps related to the long prodromal phase preceding overt disease—has not been very revealing. A number of viruses are reportedly diabetogenic, and islet-cell antibodies have been described following viral infection (74, 75). The only environmental factor clearly associated with Type-I diabetes of humans is congenital rubella infection, which also increases the incidence of a series of autoimmune disorders (e.g. thyroiditis). Increased prevalence of Type-I diabetes has been reported in DR3 positive patients with a history of congenital rubella infection (76). MHC genes involved in susceptibility to Type-I diabetes may thus be necessary for this viral pathogenic effect. The manner by which congenital rubella increases the incidence of diabetes is not known. One hypothesis is that the rubella virus leads to tissue destruction by infecting multiple target tissues (islets, thyroid). Alternatively persistent abnormalities of T-cell subsets have been described in a group of patients with a history of congenital rubella infection (77). Such T-cell abnormalities may contribute to diabetes. In addition, Horn reported that the sequence of E1 protein of rubella virus contains at position 261 a segment of five amino acids (GPPAA) identical to a segment encoded by the IDDM-associated DQ3.2 allele (78). There is also a homology with the Epstein Barr virus (EBV) genome. Horn suggests that a shared peptide could serve as an autoantigenic target for a cross-reactive immune response to the pathogen.

A Coxsackie B4 (CB4) virus was isolated from the pancreas of a child

who died from a viral encephalitis and diabetic ketoacidosis (79). Later reports of the pathological findings in the child's pancreas indicate that β cell destruction may have preceded the virus infection (80). Recently it has been shown that infection by E2 strain of CB4 virus induces long-term hyperglycemia and increases 64k islet-cell antigen expression in CD1 and SJL/1 mice, suggesting a role of this viral infection in autoimmunity (81). In contrast Ji-Won Yoon and colleagues did not find evidence of immune involvement in the induction of diabetes by encephalomyocarditis (EMC-D) virus (82). Coxsackie-B–virus specific IgM has been detected frequently in newly diagnosed insulin-dependent diabetics (83, 84), but a positive association has not been found with islet cell–related autoantibodies (85).

Viral infections have the potential not only to induce diabetes but also to prevent diabetes. Infecting both BB rats and NOD mice with lymphocytic choriomeningitis viruses (LCMV) decreased the development of diabetes (86). Another factor in animal models capable of decreasing diabetes is dietary manipulation. Elliot and Martin found that animals fed a diet containing a mixture of amino acids instead of proteins reduced diabetes incidence, and the addition of milk proteins to this diet increased the incidence (87). Cow's milk proteins and wheat gluten have been reported to be important for full expression of diabetes in BB rats. Recently changes in diet have been shown to decrease the intensity of class I–MHC products on islet cells associated with a decrease of diabetes incidence (88).

There is some circumstantial evidence suggesting a possible role of diet in the development of human insulin-dependent diabetes. An inverse correlation has been found between breast feeding and IDDM in childhood in Denmark (89), and high concentrations of N-nitroso compounds, present in smoked mutton, are speculated to be associated with seasonal changes in incidence rates for Type-I diabetes (90).

Insulin-dependent diabetes can be induced by several drugs. High doses of streptozotocin or alloxan (91, 92) produce a rapid destruction of the islet β cells in rat or mouse. Multiple small "sub-diabetogenic" doses of streptozotocin cause insulitis with delayed but progressive hyperglycemia (93). Drugs such as penicillamine (94), hydralazine (95) or methimazole (96) induce the production of insulin autoantibodies, without (to date) reports of the induction of diabetes.

The most suggestive evidence for an important environmental factor(s) influencing the development of diabetes of human is an increase in diabetes incidence which has been observed in several countries over the past several decades. This change in incidence exceeds that expected for changes in gene pool.

An alternative hypothesis to that of differential environmental exposure for discordance of identical twins is suggested by the explanation of genetic

penetrance of hereditary retinoblastoma (97). For hereditary retinoblastoma, somatic mutations rather than specific environmental factors underlie the development of disease in some but not all genetically susceptible individuals. The testing of such a hypothesis for diabetes will almost certainly require the definition of susceptibility genes, and perhaps an understanding of which cell is genetically affected (e.g. macrophages, T cells, or islets). Chromosomal dysfunction (e.g. Down's syndrome) is associated with Type-I diabetes and a series of autoimmune diseases (98). The gene responsible for the autoimmunity in these patients is unknown, but genes determining the level of interferon receptors map to the long arm of chromosome 21.

In addition to somatic mutations twins will also differ secondary to immunoglobulin and T cell receptor rearrangements (99). These "random" differences may also influence disease penetrance.

WHAT ARE THE TARGET ANTIGENS?

At present there is little information concerning specific T-cell responses to potential autoantigens. This may change rapidly as T-cell lines capable of transferring disease in the NOD mouse are developed, but autoantigens are currently defined by antibody reactivity. A series of autoantibodies have been identified including antibodies to insulin, a "cytoplasmic" islet antigen detected on frozen sections which we have evidence is a ganglioside, a 64K protein, an antigen expressed at the secretory pole of rat insulinoma cells, and a series of antigens beginning to be identified with Western blots.

With such a series of autoantibodies, three questions can be asked concerning the importance of target antigens. Which target antigen (*a*) is essential for the autoimmune process, (*b*) may function as an immunogen, and (*c*) is secondarily recognized by the immune system during islet destruction? At present the primacy of the immune response to any given antigen is not known. The central importance of a given response will probably be proven only when immunomodulation to block response to a specific antigen prevents activation of autoimmunity or interrupts ongoing autoimmune β cell destruction.

For example we are pursuing the hypothesis that an immune response (probably T cell–mediated) directed against insulin may be central to the development of Type-I diabetes. This working hypothesis is based on;

1. Studies performed more than a decade ago which indicated that animals immunized with insulin developed insulitis and rarely diabetes (100, 101).

2. The correlation of the level of insulin autoantibodies (but not other diabetes-associated autoantibodies) with the age at which Type-I diabetes develops and the rate of the progression of cytoplasmic islet-cell antibody-positive relatives to overt diabetes.

3. The expression of insulin on the surface of β cells as detected with flow cytometry and electron microscopy (102).

Arguments against this hypothesis include;

1. Anti-insulin antibodies in mice can be generated following transplantation of allogenic pituitary cells induced to synthesize homologous insulin with a transfected insulin gene (103). These mice producing antibodies to "mouse" insulin do not in general develop diabetes or insulitis.

2. The majority of older children and adults developing Type-I diabetes, and (in particular individuals slowly developing Type-I diabetes), do not express anti-insulin antibodies greater than normal controls do.

Antibodies that bind the insulin molecule (IAA) are detected in a new onset Type-I diabetic prior to insulin therapy (104, 105), and even years before the onset of diabetes (104, 106–109). Two general types of assay have been used to measure anti-insulin antibodies: fluid phase radioassays with 125-I labelled insulin (RIA) and enzyme-linked immunosorbent assays (ELISA) where insulin is bound to plastic wells. Recent workshop comparisons indicate that these two assay formats often detect qualitatively different antibodies. In particular anti-insulin antibodies, determined by ELISA, appear to have limited diabetes-related specificity in terms of predicting diabetes development.

As determined by RIA the insulin autoantibodies are of extremely high affinity (data unpublished) and low capacity and are homogenous in terms of the region of the insulin molecule recognized, with similar binding between human, porcine, and bovine insulin, but no reactivity with guinea pig or fish insulin (110).

Up to 53% of new onset Type-I diabetics have insulin autoantibodies (IAA) according to radioassays, and 39% have such, using an ELISA method (104, 111, 112). Using a radioimmunoassay we detect IAA in 53% of the ICA+ first degree relatives, in contrast to 2.7% of ICA− relatives (111). An inverse correlation has been reported between IAA and age onset of diabetes utilizing radioassays (109, 113–115). Using an ELISA assay Wilkin and coworkers detect IAA in 47% of long-term discordant identical twins of Type-I diabetes (104), indicating that this activity is not very predictive for the development of diabetes, as less than 5% of long-term discordant twins develop diabetes.

The level of insulin autoantibodies fluctuates within characteristic ranges for different prediabetics, and in our studies these levels correlate with the

rate of progression to overt diabetes. NOD mice also produce low levels of anti-"insulin" autoantibodies, and those mice with the highest levels are more likely to develop overt diabetes (116).

Recently we reported that rejection of cells expressing a transfected insulin gene, in mouse, is associated with production of insulin autoantibodies (103). Thus, IAA before diabetes may reflect the immune destruction of islet cells with secondary production of anti-insulin antibodies. Higher levels of insulin autoantibodies may be associated with a more aggressive autoimmune process. Alternatively, "cross-reactivity" has been reported for NOD mouse antibodies between insulin and a retroviral antigen p73 expressed in islets (117).

Autoantibodies against pancreatic islet-cell antigens, as measured with frozen sections of pancreas or living islet cells, have been described in detail (118, 119). To standardize the measurement of cytoplasmic islet cell antibodies (ICA), several international workshops have now compared the results of analysis of identical sera by multiple laboratories. Large variations were seen among laboratories, in both assay sensitivity and specificity. In these workshops most laboratories used frozen sections of human pancreas and indirect immunofluorescence to detect the binding of ICA. In our recent studies we have utilized frozen sections of rat pancreas (120). As a result of these standardization efforts, the expression of ICA results—in terms of dilutions of standard sera (JDF units)—has reduced interlaboratory variation.

Multiple groups have reported ICA in over 60% of patients with recently diagnosed Type-I diabetes (121–125) and in first degree relatives developing diabetes (126, 127). The prevalence of ICA in "normal" school children varies between 0.4–0.9% (128, 129). ICA in the serum of Type-I diabetic patients declines following diagnosis of diabetes (125). One in 50 of first degree relatives of a Type-I diabetic express high titer islet cell antibodies (>80 JDF units) and have approximately an 8% per year risk of developing diabetes (111).

The autoantigen to which ICA are directed has not been fully characterized. Our evidence suggests that it has the properties of a glycolipid, containing sialic acid, that is similar but not identical to the antigens reacting with anti-islet, anti-ganglioside monoclonals 3G5, A2B5, and R2D6. Further, islet cell antibody binding is inhibited by a glycolipid extract migrating as a monosido-ganglioside from human pancreas (130, 131). Gillard and coworkers have reported antibodies reacting with GT3 (132). In addition, Appel & Dotta have recently demonstrated that suppression of pancreatic islet activity with a transplantable islet tumor results in loss of pancreatic ICA antigen expression and coordinate loss of a specific ganglioside on TLC migrating between GM2 and GM1 (133, 134).

Cytoplasmic islet cell antibodies reacting with frozen sections have not been reproducibly found in sera from BB rats or NOD mice. Islet cell surface antibodies have been found in BB rats (135, 136), and recently Reddy and colleagues detected ICA in NOD mouse sera using Bouin's fixed pancreas sections (137).

Antibodies directed to a human islet cell protein of 64 kd have been detected in sera from the majority Type-I diabetics as well as from first degree relatives developing diabetes (138, 139). Similar antibodies have been found in sera from BB rats (140) and from NOD mice (141). Antibodies to this protein are detected with S35 labeling of isolated islets followed by immunoprecipitation and polyacrylamide gel electrophoresis. The sequence of this protein is currently unknown, but the molecule appears to be restricted to β cells (139). It is not clear whether the molecule is expressed on the islet cell surface (142).

Recently, Dotta and colleagues described an antibody reacting with a transplantable rat islet tumor in sera of patients with Type-I diabetes and NOD mouse. These antibodies react with rat insulinoma tissue (RINm38) at the secretory pole of the cells, and they have been detected in less than 20% of new onset Type-I diabetics, but in 98% of NOD mice (less than 6 months old). Enzymatic sensitivity studies suggest that the polar antigen is a protein. In crosses of NOD mice with normal strains, antipolar antibodies are inherited in an autosomal recessive pattern, and they are linked neither to the MHC nor to the Thy-1 locus (143).

A series of monoclonal antibody probes directed towards islet endocrine cells have been developed (144–146). The majority of these monoclonals react with neuroendocrine cells. Utilizing these antibodies several molecules have been identified and biochemically characterized. Some of them recognize islet cell proteins while others recognize glycolipid islet cell antigens. Using "Western" immunoblotting methods, additional potential autoantigens have been described (147).

WHAT ARE THE EFFECTOR CELLS/ MOLECULES LEADING TO DIABETES?

Different candidate effector systems (e.g. cytotoxic antibodies, T lymphocytes, natural killer (NK) cells, macrophages, or lymphokines have been implicated in the pathogenesis of Type-I diabetes.

1. Islet cell surface antibodies (ICSA) are detected frequently in sera of Type I-diabetic patients (119), and they are specific for insulin producing cells (148, 149). Several reports have shown that sera from diabetic patients containing ICSA may alter β cell function and be cytotoxic specifically to

the β cell in the presence of complement (149–152). Although it is not proven that such antibodies can induce diabetes, in vivo passive transfer of immunoglobulins, containing ICSA, from Type-I diabetics to normal mice alter β cell function (153). First degree relatives may have these antibodies, which are also cytotoxic for β cells (149). Despite a large number of studies concerning antisurface antibodies, the disease specificity of the assays remains controversial. For example, using rat islet tumor cells, Cavender found complement-dependent cytotoxicity with diabetic as with control sera (154). Additional evidence against a role for antibodies mediating β-cell destruction is the lack of diabetes in infants of mothers who are positive for anti-islet cell antibody and who develop diabetes during pregnancy. Transplacental passage of anti-islet antibodies can be demonstrated in cord blood of newborn infants of mothers positive for islet cell antibodies.

2. A large body of data, particularly from studies of the BB rat and NOD mouse, indicate that Type-I diabetes is a T-cell mediated disease. The mononuclear infiltrate of human pancreatic islets first suggested a clear role for autoimmunity in Type-I diabetes. The presence of T cells in this infiltrate and the increased levels of activated (Ia+) T cells in the circulation of new onset Type-I diabetes patients (155) and islet lesions support a role of activated T cells in the pathogenesis of the disease. T lymphocytes from diabetic individuals can inhibit glucose and theophylline induced insulin release from murine islets (156, 157). Other investigators have reported that circulating lymphocytes from diabetics may be cytotoxic to rat islet cells (158). CD8-positive T cells formed the majority of infiltrating lymphocytes of a pancreas in a child with new onset diabetes (159).

Multiples studied provide evidence that T cells are critical to the process of β cell destruction in animal models. Neonatal thymectomy prevents diabetes in BB rats (160) and in NOD mice (161). Transfer of spleen cells from diabetic mice into NOD mice, older than 6 weeks and previously sublethally irradiated, induces the onset of overt diabetes (162). Bendelac and colleagues transferred diabetes to neonatal NOD mice (163) and demonstrated that diabetes transfer is mediated by both L3T4 and Lyt2 T cell (164, 165), without involvement of B lymphocytes (165). An islet-specific CD4+ T-cell clone, derived from the NOD mouse, that proliferates and makes lymphokines in response to antigens presented by islet cells, can specifically destroy transplanted islets. Reich and coworkers have transferred diabetes with a T-cell clone derived from NOD islets (166).

L3T4+ helper T lymphocytes are the predominant T-cell subset infiltrating pancreatic islets in NOD mice (167) and in BB rats (168). Insulitis and diabetes in NOD mice may be prevented using monoclonal anti-L3T4

antibodies (167). In addition this treatment may prevent hyperglycemia following subdiabetogenic doses of streptozotocin (169) and diabetes induced by cyclophosphamide (170). Cultured BALB/c islet tissue is rejected in NOD mice unless L3T4 T lymphocytes are depleted by the administration of anti-L3T4 monoclonal antibody (171).

These reports indicate that T cells are essential for diabetes but do not exclude the possibility that T lymphocytes may recruit other cells (e.g. macrophages) as the final effectors for β cell destruction. In addition, the clonality of T cell receptors in islet destruction is not yet characterized. Our studies with NOD mouse bearing a Vβ T cell receptor transgene suggest that multiple Vβ T cell receptor chains are capable of contributing to the insulitis lesion (42).

3. Macrophages play a central role in cell-mediated immunity, including their role in initiation of immune response as antigen-presenting cells and in the production of lymphokines. Recent studies indicate that at the early stages of insulitis, both in BB rats (172–174) and in NOD mice (175), macrophages predominate; this fact suggests an important role for these cells in the initiation of the immune response. Macrophage infiltration has been reported to precede T-cell, killer, and B lymphocyte infiltration. The prevention of insulitis with silica administration which is selectively toxic to macrophages suggest that the insulitis of BB rats is dependent upon macrophages (172). In addition silica administration prevents the development of diabetes and insulitis in NOD mice both treated and untreated with cyclophosphamide (175).

NOD macrophages have recently been reported to suppress IL-2 production and the response of spleen cells to Con A. It is suggested that such suppression mediated by macrophages might initiate the generation of autoreactive helper T cell clones and activation of killer cells (176).

4. Activated macrophages incubated in vitro with islets impair glucose stimulated release of insulin (177) and are cytotoxic to mouse islets (178). It has therefore been hypothesized that cytokines released from the mononuclear infiltrate in insulitis may be important in β-cell destruction. Interleukin 1 (IL-1), from activated mononuclear cells, is cytotoxic to isolated rat and human islets (179–182). A correlation has been reported but not confirmed in subsequent studies between HLA-D genes and IL-1 production (183), involving HLA-DR2 individuals who are at low risk for IDDM and who are low monokine secretors. Other cytokines such as tumor necrosis factor, interferon gamma, and lymphotoxin may also play an important role in β cell damage (184, 185).

5. MHC class-II molecules are normally expressed in the surface of B lymphocytes, macrophages, dendritic cells, and other-antigen presenting cells (186, 187), and the molecules probably play an important role in the

anti-islet immune response, presenting antigens to T helper lymphocytes. The islet expression of class-II antigens was proposed by Bottazzo (188) to result in loss of T-cell tolerance to islets. Several reports have shown that insulin-containing cells from new onset diabetic patients at the time of autopsy may express HLA class-II antigens (159, 189). The etiology of this class-II antigen expression as well as α interferon expression in B-cells of the same autopsied pancreata is not known. It has been reported that interferon gamma may be the inducer of this class-II molecule expression. Campbell and colleagues were unable to demonstrate islet expression of class-II HLA antigens induced by interferon gamma (190) while Pujol-Borrel and coworkers induced class-II molecules in human islets cell using interferon gamma and tumor necrosis factor (TNF) and/or lymphotoxin (185). In't Veld, Pipeleers and colleagues analyzed, by electron microscopy, pancreas from both streptozotocin-treated and diabetes-prone BB rats (191, 192). Class-II antigen positive cells "containing" insulin by electron microscopy were observed to be macrophages and dendritic cells (192). Transgenic mice expressing I-A on islet cell can be normoglycemic (69), or if they became diabetic, the disease does not show involvement of the immune system (68, 193).

NATURAL HISTORY AND PREDICTION OF TYPE-I DIABETES

With a long prodromal phase of immunologic and subsequent endocrinologic abnormalities preceding overt Type-I diabetes, the possibility of predicting diabetes is being studied (111, 123, 126, 128, 135, 194–199). A major impetus for the prediction of diabetes is the realization that with accurate prediction, coupled with acceptable immunomodulatory therapy, diabetes may be preventable. In addition, the natural history of the development of diabetes and the manner in which predictive models can be expressed (e.g. linear predictive models) must be accounted for in any discussion of pathogenesis. There are at least two general hypotheses concerning the natural history of Type-I diabetes:

1. Autoimmunity and β-cell destruction vary markedly over time, with either a series of destructive episodes and/or accelerated autoimmunity at the time of onset of overt diabetes.
2. The autoimmune process once activated (as evidenced by specific autoantibodies in high titer) results in relatively linear β-cell destruction over long periods of time prior to the development of overt diabetes.

These two hypotheses have very different implications concerning the ability to predict the development of diabetes, and it is in the context of diabetes prediction that we shall contrast the two hypotheses.

Evidence favoring the first hypothesis includes (*a*) the very rapid (measured in months) destruction of β cells when "normal" pancreas is transplanted in the absence of immunotherapy from an identical twin to a diabetic twin mate (200), (*b*) the observation that induction of remissions when insulin is not required occurs with immunotherapy in new onset diabetics and is dependent on rapid (less than 6 weeks from diabetes diagnosis) institution of immunotherapy (201), (*c*) reports of fluctuating anti-islet antibodies (202, 203), and (*d*) reports of subtle abnormalities of endocrinologic function in normal first degree relatives and twins of Type-I diabetics (202).

Evidence favoring the second hypothesis includes (*a*) the general observation that the prodromal phase of immunologic abnormalities is usually very long (documented in some patients to exceed a decade) in pre–Type I diabetics (122, 126, 204). This is in marked contrast to identical twin transplants where an anamnestic immune response may result in more rapid β-cell destruction than did the original process. (*b*) With immunologic assays of high specificity (e.g. cytoplasmic islet cell antibodies greater than 80 JDF units or high levels of anti-insulin autoantibodies measured with radioassays), there is general consensus that the antibodies are consistently positive, and there is relatively little fluctuation. There is also a consensus that low titers of anti-islet antibodies (e.g. 20 JDF units) often are found to "fluctuate," questionably greater than the interassay variation of the assay utilized (194). In studies of identical twins and first degree relatives of Type-I diabetics we have yet to document transient high titer islet cell antibodies, and in less than 5% of relatives have we observed transient anti-insulin antibodies (205).

The existence of a quantitative assay such as that provided by radioassays for anti-insulin autoantibodies has allowed detailed study of autoantibody fluctuation. Anti-insulin antibodies of given prediabetics fluctuate over a restricted range, and the initial level of anti-insulin antibodies is very predictive of subsequent values.

We proposed a linear destruction hypothesis based on the ability in most prediabetics (but not all relatives positive for islet cell antibodies), to follow progressive loss of first phase insulin secretion prior to the development of overt diabetes (206, 207). Following such progressive loss of insulin secretion on intravenous glucose tolerance testing, subclinical elevations of glucose are seen in the majority of relatives within 1.5 years of diabetes onset. In studies of antibody negative identical twins of Type-I diabetics, we have not found subclinical abnormalities of oral glucose tolerance or intravenous glucose tolerance.

The most direct evidence that the process leading to Type-I diabetes can be approximated by a linear model (208) is, we believe, the linear relationship between two independent variables—first phase insulin secretion and the level of insulin autoantibodies (CIAA) (108), and the time to onset of overt diabetes of first degree relatives that are islet cell antibody positive. This relationship based on linear regression can be expressed as:

$$\text{Years to diabetes} = 2.2 + 0.017\,(\text{IVGTT insulin}) - 0.007\,(\text{CIAA value}),$$

where

$$N = 14,\ R = 0.76,\ \text{and}\ p < 0.001.$$

The predictive model gives relatively broad (95%) confidence limits for predicted time of diabetes onset, with confidence limits depending on the values for IVGTT or anti-insulin antibodies predicts the development of diabetes in short time periods (e.g. < 1 year) or in four year intervals (e.g. 2 to 6 years.

Since it was generated the model has been applied to an additional nine relatives who were followed to the development of diabetes with similar results. The predictions are also consistent with lack of diabetes in our ICA+, currently nondiabetic relatives who have not reached the predicted 95% upper limit of time for diabetes onset. The initial values for IVGTT insulin and level of anti-insulin autoantibodies of antibody positive relatives, or follow-up measurements, can be used in the predictive model with similar results. This suggests that overall, the progression to diabetes is determined years in advance of diabetes onset. It is currently not clear why the concentration of anti-insulin autoantibodies correlates with time to diabetes as other immunologic abnormalities such as the titer of anti-cytoplasmic islet cell antibodies do not correlate either with the level of anti-insulin antibodies or time to diabetes onset. In a similar manner the level of anti-insulin autoantibodies but not the level of cytoplasmic islet cell antibodies correlates with the age at which Type-I diabetes develops (113–116). Infants developing diabetes before age 5 characteristically have levels of anti-insulin autoantibodies 20 to 200 times greater than children developing Type-I diabetes after age 15 (209).

IMMUNOMODULATION/IMMUNOTHERAPY

A long prediabetic period with immunologic abnormalities and progressive β cell destruction suggests it may be possible to halt pancreatic β cells loss with immune intervention.

Multiple interventions can prevent diabetes in animals models. Bone marrow transplantation (210, 211), lymphocytic transfusions (212), neonatal thymectomy (161, 160), monoclonal antibodies to T cells (213) or against class-II MHC (164), and drugs such as cyclosporin A (214–216, 218) and nicotinamide have been used to prevent the onset of diabetes in NOD mice and BB rats (217). Similarly insulin therapy for β cell rest (219) or elimination of some proteins from the diet (87, 88, 220) decreased the incidence of diabetes in both animal models. Lymphocytic choriomeningitis virus infection reduces mononuclear islet cell infiltration and decreases the rate of diabetes in BB rats (86).

In human Type-I diabetes, immunomodulatory and immunosuppressive agents have been unfortunately less effective. Levamisole (221), plasmapheresis (222), gammaglobulins (223), or interferon fail to induce remission of the disease. Preliminary reports with nicotinamide, in human Type-I diabetes, indicate that it may increase the rate of remissions (224, 225), but in our experience, it did not prevent the progression to clinical diabetes in three ICA+ prediabetics (226).

To date immunosuppressive agents have been employed in humans in overt diabetics. Remissions induced by azathioprine unfortunately were not maintained (227, 228). Azathioprine may prove to be more effective when used with other immunosuppressors (229).

The most promising results have been achieved with cyclosporin A. Randomized studies show the efficacy of cyclosporin in maintaining remission of diabetes for one year in approximately 30% to 60% of new onset diabetics. Remissions, however, are almost invariably lost with three years of follow-up. Loss of non-insulin requiring remission while continuing cyclosporin is associated with maintenance of continued insulin secretion (e.g. C-peptide secretion). Thus cyclosporine may be preventing further β cell destruction, but so few β cells are present at onset of diabetes that they cannot maintain a nondiabetic state over time (230–233). Predictors that patients will develop a remission with cyclosporin are: a short duration of the disease, absence of ketoacidosis, weight loss, and lower glycosylated hemoglobin levels (233). Three factors suggest that the degree of β cell destruction determines remission. Depending on dosage and blood levels, cyclosporine A is associated with nephrotoxicity. In the presence of an inability to maintain a nondiabetic state, this fact makes its use after onset of diabetes problematic.

Immunosuppression may be more effective before clinical diabetes when greater β cell mass is still present. Studies of preventive therapy depend on identifying extremely high risk subjects. Ability to predict diabetes combined with improved immunomodulatory therapy will, we hope, lead to the prevention of Type-I diabetes.

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Hsp60 Peptide Therapy of NOD Mouse Diabetes Induces a Th2 Cytokine Burst and Downregulates Autoimmunity to Various β-Cell Antigens

Dana Elias, Aviram Meilin, Vitaly Ablamunits, Ohad S. Birk, Pnina Carmi, Stephanie Könen-Waisman, and Irun R. Cohen

A peptide of the human 60-kDa heat-shock protein (hsp60), designated p277, was found to be useful as a therapeutic agent to arrest the autoimmune process responsible for diabetes in nonobese diabetic (NOD) mice. The effectiveness of peptide treatment was associated with the induction of peptide-specific antibodies of the IgG1 but not of the IgG2a isotype, suggesting the possibility that a Th2-type response may have been induced. We now report that the effectiveness of p277 treatment is associated with the transient activation of anti-p277 splenic T-cells that produce the Th2 cytokines interleukin-4 (IL-4) and IL-10. The Th2 response to p277 was associated with reduced Th1-type autoimmunity to hsp60 and to two other target antigens associated with diabetes: GAD and insulin. The Th2 shift appeared to be relatively specific; spontaneous T-cell reactivity to a bacterial antigen peptide remained in the Th1 mode in the p277-treated mice. Moreover, treatment with the bacterial peptide did not induce a change in cytokine profile, and it did not affect progression of the disease. Thus, effective peptide treatment of the diabetogenic process associated with the induction of antibodies may be explained by selective and transient activation of Th2 autoimmune reactivity.
Diabetes 46:758–764, 1997

The spontaneous autoimmune process resulting in diabetes in the nonobese diabetic (NOD) mouse is first detectable as mild insulitis beginning at about 1 month of age. In most female mice, insulitis progresses to a penetrating intra-islet infiltrate that leads to β-cell damage and overt IDDM that surfaces at about 4–5 months of age (1). Adoptive transfer experiments have led to the conclusion that autoimmune T-cells of both $CD4^+$ and $CD8^+$ subsets are involved in the disease process (2). Howev r, $CD4^+$ T-cells on their own have been reported to be able to trigger diabetes (3). Two characteristics of the autoimmune $CD4^+$ T-cells seem to be important: the type of cytokines produced by the T-cells and the antigens they recognize. Mouse $CD4^+$ T-cells can be divided into two functional groups by the cytokines they secrete when activated (4): Th1 cells secrete interleukin-2 (IL-2), which induces T-cell proliferation, and proinflammatory cytokines such as γ-interferon (IFN-γ), which mediates tissue inflammation and stimulates B-cells to produce IgG2a antibodies; Th2 cells, in contrast, secrete cytokines such as IL-4 and IL-10 that can downregulate Th1 cells. IL-4 helps B-cells secrete antibodies of the IgG1 isotype and suppresses the production of Th1 proinflammatory cytokines (5). IL-10 indirectly inhibits Th1 activation by affecting antigen-presentation and proinflammatory cytokine production by macrophages (6). Because Th1-type, but not Th2-type, T-cell clones can transfer diabetes (7,8), it has been suggested that the diabetogenic autoimmune process might be aborted by inducing a shift in the relevant autoimmune T-cell activity from the Th1 type to the Th2 type (9).

With regard to target antigens, three defined antigens (GAD, insulin, hsp60) have been reported to influence the diabetogenic process (10). The administration of GAD by intrathymic injection (11), nasal inhalation (12), or intravenous injection at 3 weeks of age (13) was found to inhibit the development of T-cell reactivity to GAD and to other self-antigens and to prevent diabetes. Administration of insulin to young NOD mice was also reported to affect the development of disease (14). In addition to these antigens, we have shown that autoimmunity to hsp60 has a functional role in diabetes: NOD mice spontaneously develop T-cells responsive to the hsp60 peptide p277, and these T-cells can adoptively transfer diabetes or, when attenuated, can vaccinate mice against diabetes (15). A single, subcutaneous administration of peptide p277 in oil either early at 4–6 weeks of age (15) or very late at 12–17 weeks in the autoimmune process can arrest the disease (16,17).

Peptide p277 was also found to influence toxin-induced diabetes. Mice of the C57BL/*KsJ* strain can be induced to develop a type of autoimmune diabetes about 3 months after administration of a very low dose of the β-cell toxin, streptozotocin (18). This form of diabetes could also be treated with peptide p277 administered after the toxic insult. In contrast to p277 treatment, the treatment of mice with an immunogenic GAD peptide failed to arrest the development

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cpm, counts per minute; Con A, concanavalin A; ELISA, enzyme-linked immunosorbent assay; hsp60, 60-kDa heat-shock protein; IL, interleukin; IFA, incomplete Freund's adjuvant; IFN-γ, γ-interferon; mAb, monoclonal antibodies; MT, *Mycobacterium* tuberculosis; NOD, nonobese diabetic; OD, optical density; PBS, phosphate-buffered saline; TGF-β, transforming growth factor-β.

of diabetes (19). Interestingly, the effectiveness of p277 was associated with the induction of specific antibodies of the IgG1 and IgG2b isotypes, suggesting the possibility of a shift in the cytokine profile (19).

The present study was done to investigate whether a change in T-cell cytokine secretion was indeed associated with the response to p277 treatment, and whether the effect of p277 treatment might spread to the responses to other autoantigens.

RESEARCH DESIGN AND METHODS

Mice. Inbred NOD/Lt mice were raised and maintained under specific pathogen-free (SPF) conditions at the animal breeding center of this institute from a breeding nucleus originally provided by Dr. E. Leiter (Jackson ImmunoResearch Laboratories, Bar Harbor, ME). The onset of clinical IDDM in the female mice in this colony begins at about 3.5 months of age and reaches a cumulative incidence of 80% or greater by 8 months of age. Female mice were used in these studies.

Peptides and antigens. Peptides were synthesized by standard Fmoc using an automated ABIMED synthesizer AMS422 (Langenfeld, Germany) as described (16,17). The peptides were purified by reverse-phase high-performance liquid chromatography (HPLC), and their compositions were confirmed by amino acid analysis. The sequence of p277 used in the experiments shown here was VLGGGVALLRVIPALDSLTPANED. This peptide is substituted at positions 6 and 11 with valine (Val) in place of the cystein (Cys) in the native sequence. Substitution of the two Cys residues by Val enhances greatly the stability of the peptide without affecting its immunological activity: the V-substituted peptide is completely cross-reactive with the native peptide by T-cell and antibody assays, and both peptides have the same therapeutic effect on diabetes (D.A., A.M., V.A., O.S.B., P.C., S.K.-W., I.R.C., unpublished observations). All the effects shown in this paper have been repeated with either variant of p277. The sequence of *Mycobacterium* tuberculosis (MT)-p278 is EGDEATGANIVKVALEA. The sequence of GAD peptide 34 (residues 500–528; GADp34) is IPPSLRTLEDNEERMSRLSK (11). Bovine insulin was purchased from Sigma Chemical (St. Louis, MO). Recombinant hsp60 (15) and recombinant GAD65 (13) were prepared as described. The GAD65 gene was kindly supplied by Dr. D. Kaufman, (UCLA, Los Angeles, CA). Concanavalin A (Con A) was purchased from Sigma Israel Chemicals.

Cytokine assays. At various time points, groups of five treated or naive mice were killed, their spleens were removed, and the spleen cells were pooled. The spleen cells were incubated in triplicate with medium alone or with peptides (10 μg/ml) as described (15). Supernatants were collected at 24 h (for IL-2 and IL-4 secretion) and at 72 h (for IL-10 and IFN-γ secretion). The presence of the cytokines in the culture supernatants was quantitated by enzyme-linked immunosorbent assay (ELISA) using Pharmingen paired antibodies (purchased from Pharmingen, San Diego, CA), according to the Pharmingen cytokine ELISA protocol. Pharmingen recombinant mouse cytokines were used as standards for calibration curves. Briefly, flat-bottom 96-well microtiter plates were coated with rat anti-mouse cytokine monoclonal antibodies (mAbs) for 18 h at 4°C, and the culture supernatants or recombinant mouse cytokines were added for 18 h at 4°C. The plates were washed, and biotinylated rat anti-mouse cytokine mAbs were added for 45 min at room temperature, then extensively washed, and avidin-alkaline phosphatase was added for 30 min. The plates were washed, a chromogen substrate was added, and samples were read at 405 nm in an ELISA reader (Anthos Labtec, Salzburg, Austria). The concentrations of cytokines are shown as the mean picogram per milliliter or nanogram per milliliter derived from calibration curves using recombinant cytokines as standards. The lower limits of ELISA sensitivity for the reagents used in the experiment shown in Fig. 4 were as follows: IL-4, 0.25 pg/ml; IL-2, 1 pg/ml; IL-10, 1 pg/ml; IFN-γ, 100 pg/ml. The lower limits of sensitivity for the reagents used in Table 3 were as follows: IL-4, 0.2 ng/ml; IL-10, 1 ng/ml. The differences in sensitivity between the experiments were due to biologic differences in the different cytokine standards used in these experiments.

Peptide treatment and T-cell proliferation. Groups of NOD mice were treated at the age of 12 weeks with 100 μg of peptides p277 or MT-p278 emulsified in oil (incomplete Freund's adjuvant [IFA]; Difco, Detroit, MI) or with phosphate-buffered saline (PBS) emulsified in oil as described (16,17). Five weeks later, the spleens of some of the mice were removed and the T-cell proliferative responses were assayed in vitro to the T-cell mitogen Con A (1.25 μg/ml; Sigma) or to various peptides (10 μg/ml) using a standard assay (20). Dose-response curves were done using concentrations of peptides up to 25 μg/ml (not shown). The concentration of 10 μg/ml was chosen to illustrate the results because this concentration produced the optimum response. The T-cell responses were detected by the incorporation of [^{3}H]thymidine added to the wells in quadruplicate cultures for the last 18 h of a 3-day culture. The stimulation index (SI) was computed as the ratio of the mean counts per minute (cpm) of antigen-containing wells to control wells cultured without antigens or Con A. The standard deviations from the mean cpm were always <10%. Background cpm, in the absence of antigens, was 800–1,500 cpm. Some of the treated mice were scored for the development of diabetes by 8 months of age as described (16).

Antibody assays. Groups of NOD mice, 12 weeks old, were treated with p277 in oil or with PBS in oil. Five weeks later, the mice were bled individually and their sera were diluted up to 1:500 and tested for antibodies to recombinant GAD65 (18), to recombinant human hsp60 (20), to bovine insulin, or to peptide p277 in an ELISA assay as described (20). Briefly, 10 μg of the various antigens were applied to assay plates (Maxisorp, Nunc, Roskilde, Denmark) suitable for the binding of peptides, and the plates were incubated with the test sera. The binding of antibodies to the adherent antigens was detected using alkaline phosphatase conjugated anti-mouse IgG + IgM or isotype-specific anti-mouse IgG1, IgG2a, or IgG2b (Jackson ImmunoResearch Laboratories, West Grove, PA). A significant amount of antibody was defined as an optical density (OD) 405 nm reading of >0.25, which is 3 SD over the mean ELISA reading obtained in the sera of 10 normal BALB/c mice. The figures show the results obtained at the 1:50 dilution because this was the serum dilution that produced the highest incidence of positive antibodies for all of the antigens tested.

TABLE 1
Peptide p277 treatment of NOD diabetes is specific

Peptide treatment	Incidence of diabetes at 8 months of age	
	Normoglycemic	Hyperglycemic
None	3	97
PBS	10	90
MT-p278	17*	83*
p277	53†	47†

Data are %. Groups of 30 NOD female mice were injected subcutaneously at 12 weeks of age with peptides (100 μg) MT-p278 or p277, or with PBS emulsified in IFA. The mice were examined monthly and scored for the development of hyperglycemia by 8 months of age using a glucose analyzer as described (17). Blood glucose concentrations in the hyperglycemic mice at 8 months were all >35 mmol/l. *P = 0.71 compared to PBS-treated mice; †P = 0.006 compared to MT-p278–treated mice.

RESULTS

Specificity of p277 treatment. Table 1 shows the results of treating NOD female mice with peptides p277 or MT-p278 in IFA, or with PBS in IFA. The mice were followed until the age of 8 months and marked for the development of hyperglycemia. As we have reported earlier, the administration of p277 was associated with a significant decrease in the cumulative incidence of mice developing diabetes (47%), compared with the control-untreated or PBS-treated mice (90–97%). Treatment with the immunogenic peptide MT-p278 did not lead to a significant reduction in disease (83%). Thus, the inhibitory effect of p277 peptide treatment on the spontaneous development of diabetes was specific.

Effect of p277 treatment on autoantibodies. The development of diabetes in NOD mice has been associated with spontaneous autoantibodies to self-antigens such as hsp60 (20), GAD (13), and insulin (20). We therefore wished to see the effect of peptide p277 on these spontaneous autoantibodies and on the induction of antibodies to p277. Figure 1 shows the incidence of mice with autoantibodies in the sham-treated and p277-treated groups. The control-treated NOD mice mani-

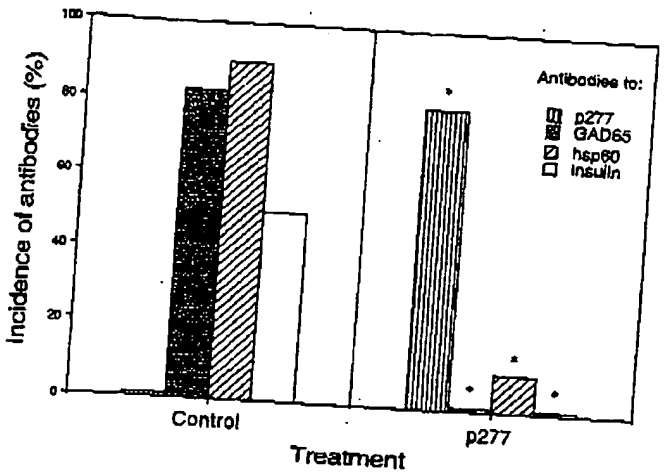

FIG. 1. Treatment with p277 induces antibodies to p277 and reduces antibodies to GAD, to insulin, and to intact hsp60. Groups of 10 NOD mice, 3 months old, were treated with p277 in oil (*A*) or with PBS in oil (*B*). Five weeks later, the mice were bled individually, and their sera, diluted 1:50, were tested for antibodies to recombinant GAD, to recombinant human hsp60, to bovine insulin, or to peptide p277 in an ELISA assay. A significant amount of antibody was defined as an OD 405-nm reading of >0.25, which is 3 SD over the mean ELISA reading obtained in the sera of 10 normal BALB/c mice. The results are shown as the incidence of mice positive for antibodies to the various antigens. A sample of actual OD 405-nm readings can be seen in Fig. 2. $^*P < 0.01$ by χ^2 test.

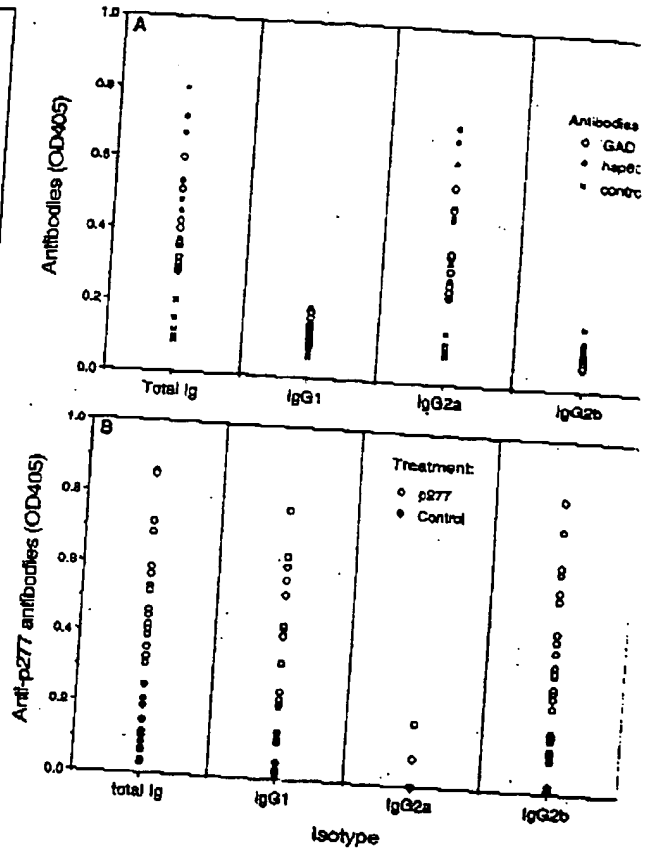

FIG. 2. Antibody isotypes before and after p277 therapy. Groups of NOD mice, 3 months old, were treated with p277 or with PBS in oil (control). The sera of individual mice in each treatment group were assayed 5 weeks after treatment for the isotypes of their antibodies to intact hsp60 or to GAD before treatment (*A*) or for the isotypes of their antibodies to p277 after treatment (*B*) (12–15 mice per group). The antibody isotypes were detected using an ELISA assay with isotype-specific developing antibody reagents. Because of superimposition of the circles, the three circles in the IgG2a column in Fig. 2*B* represent 12 mice.

fested a high incidence of antibodies to GAD (80%) and to hsp60 (90%), and a moderate incidence of anti-insulin antibodies (50%); there were no spontaneous antibodies to peptide p277. Similar results were obtained in untreated control NOD mice (not shown). In contrast, the p277-treated mice showed a marked increase (80%) in the incidence of antibodies to p277 ($P < 0.01$). Nevertheless, the p277-treated mice showed a significant reduction in the incidence of autoantibodies to GAD, whole hsp60, and insulin ($P < 0.01$).

Analysis of the isotypes of the antibodies produced before and after p277 therapy were done to extend the observation that effective treatment might be associated with certain antibody isotypes (19). Figure 2*A* shows that the spontaneous autoantibodies to GAD and to whole hsp60, which were present before treatment with p277, were of the IgG2a class, antibodies dependent on T-cells of the Th1 type that secrete IFN-γ (21). None of the anti-GAD or anti-hsp60 antibodies were of the IgG1 isotype associated with the Th2 cytokine IL-4 (21). In contrast, analysis of the antibody isotypes of the anti-p277 antibodies induced by treatment (Fig. 2*B*) showed them to be mainly of the IgG1 and IgG2b classes. There were significantly fewer Th1-type IgG2a antibodies to p277 induced by p277 therapy ($P < 0.01$).

Effects of p277 treatment on T-cell proliferation. To test the effects of p277-peptide treatment on T-cell proliferation, spleen cells were obtained from parallel groups of p277-treated and sham-treated mice and tested at 17 weeks of age (5 weeks after treatment) for their T-cell proliferative responses to peptide p277 of hsp60, to peptide p34 of GAD (11), or to peptide MT-p278 of mycobacterial hsp60. Figure 3 shows that the sham-treated NOD mice manifested spontaneous reactivity to all three peptides and to Con A. Untreated control NOD mice manifested similar spontaneous T-cell reactivities (not shown). In contrast, the p277-treated NOD mice showed a specific fall ($P < 0.01$) in their T-cell proliferative responses to the hsp60 and GAD peptides. The control responses to MT-p278 and to Con A remained intact.

Effects of p277 treatment on cytokines. The above results indicated that p277 treatment, which modulates the autoimmune process, was associated, on the one hand, with induction of IgG1 and IgG2b peptide-specific antibodies and, on the other hand, with inhibition of spontaneous IgG2a antibodies and T-cell proliferation to other antigens. The question, therefore, was whether these immunological changes could be explained by a change in cytokine profile induced by p277.

Experiments were done to document the natural history of representative Th1 and Th2 cytokines produced by anti-p277 T-cells during progression of the autoimmune process and to detect any effects of p277-peptide or sham treatment on the cytokines produced by these spontaneously appearing anti-p277 T-cells. Table 2 shows the concentrations of a key Th1 cytokine, IFN-γ, and of two Th2 cytokines, IL-4 and IL-10, pro-

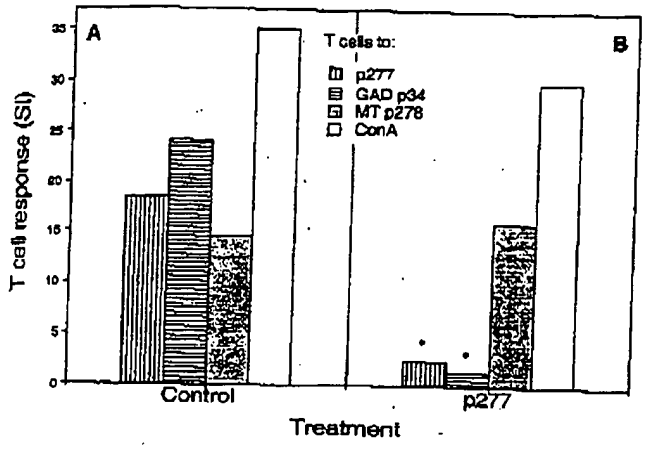

FIG. 3. Spontaneous T-cell proliferative responses to GAD and hsp60 peptides are specifically reduced by p277 therapy. Groups of NOD female mice were treated at the age of 8 months with 100 μg of peptide p277 (*A*) emulsified in mineral oil or with PBS emulsified in mineral oil (*B*) as described (16,17). Five weeks later, the spleens of the mice were removed and the T-cell proliferative responses were assayed in vitro to the T-cell mitogen Con A or to three different peptides.

duced by spleen cells activated in vitro in response to incubation with peptide p277. Groups of mice were examined for these anti-p277 cytokine responses at ages 5 weeks (onset of insulitis), 12 weeks (advanced insulitis), 17 weeks (onset of clinical diabetes), and 28 weeks (far-advanced clinical diabetes). We treated the mice at 12 weeks of age, just before the outbreak of overt diabetes. Table 2 includes the concentrations of cytokines produced by anti-p277 T-cells both before and after treatment.

Before treatment, the NOD mice manifested an appreciable level of IFN-γ responsiveness to p277 at 5 weeks, which rose sharply at 12 weeks. Sham-treated mice at 12 weeks, like untreated NOD mice, showed a decline in their anti-p277 IFN-γ production at the onset of diabetes (17 weeks) and late in the disease (28 weeks). In the absence of p277 treatment, there was no appreciable IL-4 activity produced by the anti-p277 T-cells; a rise in IL-10 activity was seen at 17 and 28 weeks. After treatment with peptide p277 at 12 weeks, however, there was an abrupt fall in IFN-γ secretion at 17 weeks and 28 weeks. In contrast to the fall in IFN-γ, there appeared sharp increases in IL-4 and IL-10 responsiveness at 17 weeks. The p277-treated mice, free of diabetes at 28 weeks of age, manifested a continued low IFN-γ response but showed a fall in their IL-4 and IL-10 responses (Table 2). Thus, arrest of the diabetogenic process induced by p277-peptide therapy was marked by a burst of Th2-type reactivity detected 5 weeks after treatment that later reverted spontaneously.

Specificity of cytokine modulation. The immunological specificity of T-cell cytokines was measured at 17 weeks (5 weeks after treatment) by comparing the cytokine profiles of the responses to p277 with those induced by peptide MT-p278 of mycobacterial hsp60. Our NOD mice show spontaneous T-cell reactivity to peptide MT-p278, but administration of the MT-p278 peptide does not affect diabetes (Table 1), so this peptide can serve as a convenient T-cell specificity control. Figures 4*A* and 4*B* show that the spleen cells of sham-treated mice secreted both IL-2 and IFN-γ upon incubation with either

TABLE 2
Cytokines produced by anti-p277 T-cells before and after treatment with peptide p277 at 12 weeks of age

	IFN-γ	IL-4	IL-10
Before treatment			
5 weeks	6	0	0
12 weeks	80	0	0
After treatment			
17 weeks			
None	60	0	0.35
Sham	50	0	0.5
p277	0.9*	12.8*	7*
28 weeks			
None	17	0	1
Sham	14	0	1.2
p277	0.6*	0.7	1.6

Data are in nanograms per milliliter. Groups of five female NOD mice were killed at various ages, and the cytokines produced in response to incubation in vitro with peptide p277 were measured. Some of the groups of mice were treated with peptide p277 (100 μg) or with PBS in IFA at the age of 12 weeks. The SE were <10% of the mean in all groups. "0" signifies a concentration below the lower level of detection; 10 p277-treated and 10 sham-treated mice were followed up to the age of 32 weeks to determine the incidence of hyperglycemia and mortality resulting from diabetes. The sham-treated mice manifested an incidence of hyperglycemia of 9/10, and 7/10 of the mice died of severe diabetes. In contrast, the p277-treated mice manifested an incidence of diabetes of 3/10 and an incidence of death of 1/10 ($P < 0.05$). *$P < 0.01$ compared with untreated or sham-treated mice.

p277, MT-p278, or the T-cell mitogen Con A. In contrast, the p277-treated mice produced significantly less IL-2 and IFN-γ in response to incubation with peptide p277 ($P < 0.01$). This reduction in Th1 cytokines was specific; the p277-treated mice maintained their IL-2 and IFN-γ cytokine responses to MT-p278. Figures 4*C* and 4*D* show the amounts of IL-10 and IL-4 produced by the spleen cells of the mice. The sham-treated mice produced negligible amounts of IL-4 in response to p277, MT-p278, or Con A, and only a small amount of IL-10 in response to p277. In contrast, there was a significant increase in IL-10 and IL-4 in response to p277 in the p277-treated mice ($P < 0.01$). A decrease in IL-2 and IFN-γ coupled with an increase in IL-10 and IL-4 indicates that the shift from Th1-like behavior to Th2-like behavior in response to p277 was immunologically specific, relative to the response to MT-p278.

To confirm the specificity of the Th2-like response to peptide p277, groups of NOD female mice were treated with PBS or with peptides MT-p278 or p277, in IFA. Five weeks later, the splenic T-cells of the mice in each group were stimulated in vitro by incubation with MT-p278 or p277 and the media were assayed for the secretion of IL-4 and IL-10. Table 3 shows that the mice that had been treated with MT-p278 showed no increase in the secretion of IL-4 or IL-10 over that of PBS-treated mice when the spleen cells were incubated with MT-p278. In contrast to the mice that had been treated with PBS or with MT-p278, the mice that had been treated with p277 showed a marked increase in IL-4 and IL-10 induced by incubation with p277. Thus, the rise in Th2 cytokine reactivity was specific for the p277 peptide.

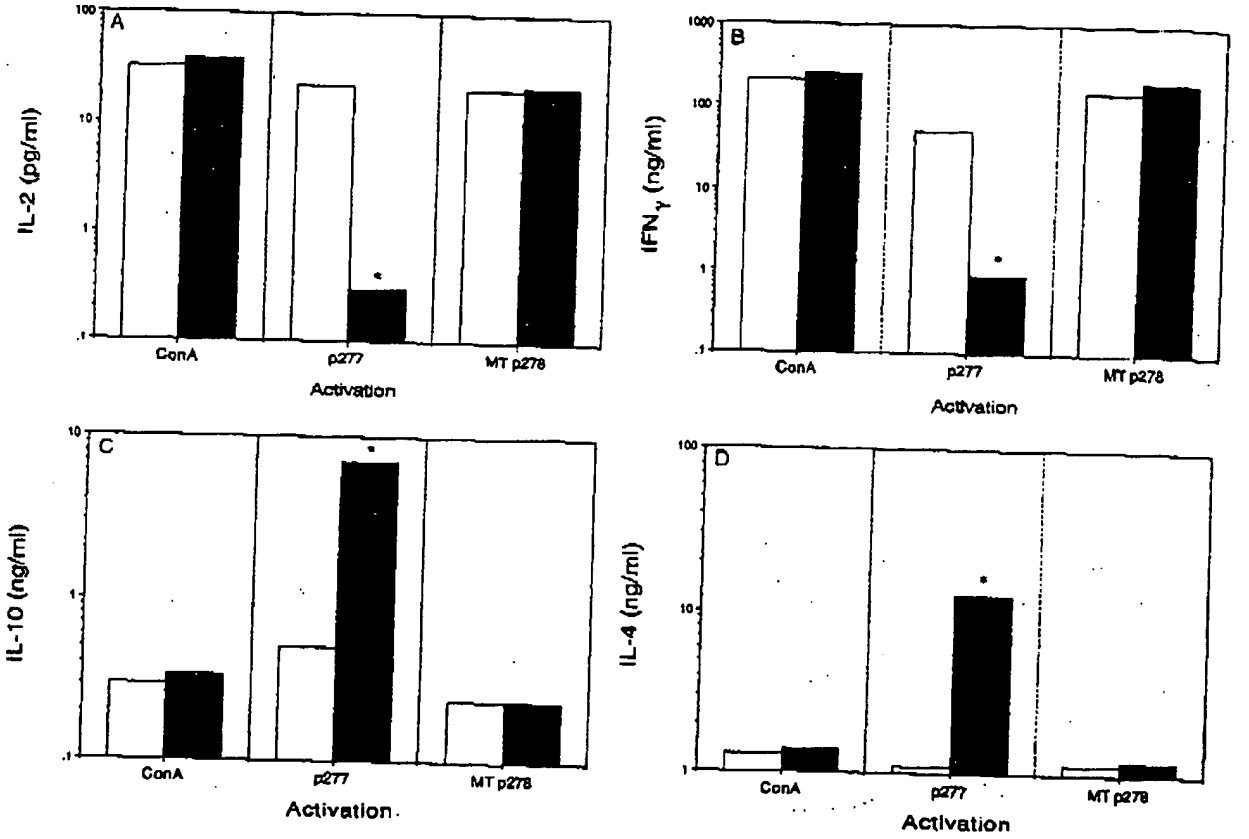

FIG. 4. Peptide p277 therapy induces a specific switch in the cytokine profile. Groups of 10 NOD mice, 3 months old, were treated with p277 in oil (solid bars) or with PBS in oil (open bars). Three weeks later, the spleens of the mice were removed and the spleen cells were pooled. The spleen cells were incubated in triplicate with Con A, p277, or MT-p278 for 24 h (for IL-2 and IL-4 secretion; [*A*, *D*]) or for 48 h (for IL-10 and IFN-γ secretion [*B*, *C*]). The presence of the cytokines in the culture supernatants was quantitated by ELISA. The concentrations of cytokines are shown as mean picogram per milliliter derived from calibration curves using recombinant cytokines as standards. The SE was <10% of the mean in each group. *$P < 0.01$ by Student's *t* test.

DISCUSSION

The experiments described here were done to learn whether the effectiveness of hsp60 peptide treatment of the autoimmune diabetogenic process in NOD mice might be associated with a change in cytokine secretion. This study was suggested by our observations that effective treatment with peptide p277 induced high titers of specific antibodies of the IgG1 isotype, thought to be induced by IL-4 (21), while immunogenic peptides that were not effective in arresting diabetes did not induce such antibodies (19). Treatment with p277 also induced peptide-specific antibodies of the IgG2b isotype (Fig. 2 [19]). The cytokines required for induction of antibodies of the IgG2b isotype are controversial; although small amounts of the "suppressor" cytokine transforming growth factor-β (TGF-β) may be required for the secretion of all IgG isotypes (22), it appears that IgG2b is induced primarily by TGF-β (21,23). However, we have yet to obtain direct evidence for the secretion of TGF-β; thus the possible involvement of this cytokine in the effect of p277 must be viewed with caution. Be that as it may, our results are compatible with the association of IgG2b with downregulation of autoimmune damage.

Autoimmune Th1 cells are thought to be the pathogenic agents in autoimmune IDDM, and Th2 cells, in contrast, are thought to be innocuous or even beneficial in halting th diabetogenic autoimmune process (9). Indeed, young NOD mice have been treated by direct injection of Th2 cytokines (24–26). Healey et al. (8) and Katz et al. (7) found that lines of IFN-γ-secreting Th1 cells could transfer diabetes, but that Th2 cell lines could not transfer the disease. However, the direct therapeutic value of Th2 cells is still not clear. Adoptive transfer of a Th2 clone was reported not to protect against diabetes (7). Moreover, a transgenic mouse hyperexpressing IL-10 in the islets was reported to develop IDDM (27). Therefore, not all Th2 cells that are targeted to the islets are beneficial, and persisting secretion of a Th2 cytokine may actually damage β-cells.

The studies reported in this paper relate to the p277 peptide of the human hsp60 molecule, which can serve as a target of diabetogenic T-cells in the NOD mouse (15). This peptide differs from the analogous hsp60 sequence in the mouse by 1 amino acid at position 455. However, the mouse and human p277 peptides appear to be completely cross-reactive for the same clone of T-cells, and administration of either peptide can halt the development of diabetes (28). Thus, the response to the human p277 sequence, even with further substitution of the two cystein residues, functions as does the self-mouse peptide.

TABLE 3
Induction of Th2 cytokines is specific for p277 treatment

Stimulation in vitro	Peptide treatment in vivo	IL-4	IL-10
MT-p278	PBS	0	0
	MT-p278	0	0
p277	PBS	0	0
	p277	13.7 ± 0.6*	8.5 ± 0.5*

Data are means ± SD and nanograms per milliliter. Groups of 10 female NOD mice, 12 weeks old, were injected subcutaneously with peptides (100 μg) MT-p278 or p277 in IFA, or with PBS in IFA. Five weeks later, the spleen cells were stimulated by incubation in vitro with peptides (10 μg/ml) MT-p278 or p277, and the culture media were assayed for the secretion of IL-4 and IL-10. "0" signifies a concentration below the lower level of detection. *$P < 0.001$ compared with mice treated with PBS or MT-p278.

Peptide p277 administered in different contexts can have different effects. Immunization to p277 covalently conjugated to an immunogenic carrier protein such as ovalbumin or bovine serum albumin emulsified in oil was found to induce hyperglycemia and insulitis in various strains of mice, including mice not known to be prone to diabetes such as C57BL/6 and C3Hcb mice (29). Immunization with whole hsp60 in oil is also diabetogenic (20). In contrast to the diabetogenic potential of p277 peptide conjugates, the administration of peptide p277 unconjugated to a carrier was found to induce arrest of the spontaneous autoimmune diabetogenic process in NOD mice (15–17). It is not yet clear how the context of administration can influence disease. Intravenous injection of p277 was reported not to induce resistance to diabetes in NOD mice (11), but inspection of the report shows that the authors injected a truncated peptide of only 12 of the 24 amino acids; this truncated peptide was found by us to have reduced effectiveness upon subcutaneous inoculation (17). In contrast to p277, administration of a strongly immunogenic mycobacterial peptide, MT-p278, did not influence the disease (Table 1). A GAD65 peptide immunogenic in NOD mice, GADp35, was also not effective in arresting the disease (D.A., A.M., V.A., O.S.B., P.C., S.K.-W., I.R.C., unpublished observations). Thus, the effect of p277 was relatively specific. In adoptive transfer experiments, T-cells from p277-treated NOD mice were able to suppress the diabetogenic activity of preformed effector cells (17). This suggested that p277 peptide treatment might influence the production of inflammatory cytokines produced by T-cells responsive to relevant antigens in the treated mice. The present study makes three points about NOD disease and p277 therapy.

First, successful treatment of the advanced autoimmune process by administration of p277 is associated with the induction of IL-4 and IL-10 responsiveness to p277 accompanied by a sharp fall in IFN-γ. The IL-4 response can explain the association of the effective peptides with the induction of IgG1 (Th2) antibodies. Thus, a pathogenic Th1-type response to p277 is replaced by a Th2-type response to p277. Since Th1 but not Th2 T-cells are diabetogenic (7,8), the shutdown of the Th1 response constitutes therapy. We find that a clone of anti-p277 T-cells can transfer diabetes when the T-cells act in a Th1 mod and produce IFN-γ, but variants of the clon that produce IL-4 rather than IFN-γ are not pathogenic and can even protect (D.A., A.M., V.A., O.S.B., P.C., S.K.-W., I.R.C., unpublished observations).

Second, the IL-4 and IL-10 responses to p277 therapy do not persist, and long-term arrest of the disease process is associated with reinstatement of a "resting" state characterized by low level production of IFN-γ by splenic anti-p277 T-cells. We do not know the mechanism of the spontaneous decline of the IL-4 and IL-10 responses to p277 some months after the peptide therapy, but it is conceivable that the IL-4 and IL-10 responses end for lack of activation after the peptid is metabolized or cleared from the body. Thus, p277 peptide therapy seems to avoid the potential danger of chronic Th2-type autoimmunity (27).

Third, resetting the cytokine response to a single epitope, such as p277, can spread to existing Th1-type antibody and T-cell responses to other autoantigens involved in the disease, such as GAD and insulin. Note that the regulation of the T-cell response phenotype was relatively specific; the spontaneous T-cell response to a bacterial peptide, MT-p278, remained in the Th1 mode in the treated mice.

The mechanism by which a single antigen may regulate the response to other antigens needs investigation. It is conceivable that GAD and the p277 peptide can mutually regulate T-cell responses by a type of "bystander suppression" in which an antigen that triggers the production of Th2 anti-inflammatory cytokines at the site of inflammation can shut off the Th1 response of other T-cells to adjacent antigens (30). Such bystander suppression could account for the ability of T-cells from p277-treated mice to suppress the adoptive cotransfer of diabetogenic T-cells (17).

The ability of hsp60 autoimmunity to regulate NOD diabetes has been confirmed in an hsp60 transgenic model in which we engineered hyperexpression of hsp60 in the thymus and elsewhere by combining transgenic mouse hsp60 with an major histocompatibility complex (MHC) class II promoter (31). The transgenic NOD mice manifested downregulation of their spontaneous proliferative responses both to p277 and to the GAD peptide GADp34, but maintained their spontaneous responses to MT-p278. The transgenic mice were resistant to the development of diabetes (31). We are presently investigating the cytokine profiles of these mice. In contrast to our results with hsp60 transgenic mice, intrathymic injection of hsp60 at 3 weeks was reported not to be effective in preventing diabetes in NOD mice (13).

In summary, p277 peptide treatment not only is associated with induction of specific IgG1 isotype antibodies, but also affects both the cytokine profile and the antigen reactivity of the collective of the T-cells involved in the process. Further work will be needed to understand in molecular terms how administration of a peptide recognized by Th1 T-cells can activate anti-peptide T-cells of the Th2 type. Be that as it may, the ultimate transition from the Th2 burst to a baseline cytokine state suggests that the potential for physiological regulation of destructive autoimmunity is programmed within the immune system (32,33); it need only be activated by a suitable signal.

ACKNOWLEDGMENTS

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PEPTIDE LIBRARY: A NOVEL APPROACH FOR THE IFICATION OF IA-2 AUTOREACTIVE EPITOPES IN TYPE 1 TES.

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and aims: the IA-2 protein is one of the major autoantigens in type 1 and aims: the IA-2 protein is one of the major autoantigens in type 1 target of autoantibodies and autoreactive T-cells in patients with islet ry. So far, the autoreactive epitopes of this molecule have been only characterized. Display of peptides on the surface of bacteriophage has idely utilized methodology for screening of cDNA libraries. Aim of our to identify IA-2 epitopes utilizing a strategy, novel for autoimmune consisting in the construction of a random priming library of the IA-2 gene ICA512bdc: a.a.256-556:650-979), displayed as a fusion to the C-terminus id protein D of bacteriophage λ. Materials and methods: the study was in three steps: 1) Construction and characterization of a lambda surface- ICA512 bdc cDNA library with the methodology of tagged random 2) affinity selection of the resulting library, followed by immunoscreening, ay and sequence analysis of positive clones using a total of 50 ICA512 bdc ew onset type 1 diabetic patients (bdcPD) and 24 healthy controls (ctrl); 3) of a radioimmunoprecipitation assay to detect autoantibodies to the selected ty-two bdcPD and twenty-five ICA512 bdc negative new-onset type 1 tients (bdcNP) , 20 ICA512bdc negative relatives of type 1 diabetic (bdcNR) and 25 ctrl were tested with these assays. Results: eleven clones ected and sequenced, among these only one (IA-2 761-964) and a ion of other two (IA-2 929-979) were used in radioimmunoprecipitation the basis of their specific reactivity with IDDM sera. All bdcPD were for IA-2 (761-964) autoantibodies, while only 74,2% (46/62) of these patients d the fragment IA-2 (929-979). All bdcNP were negative for IA-2 (929-979) bodies, but one of these patients was IA-2 (761-964) positive. No reactivity the two IA-2 constructs was detected in bdcNR and ctrl. Conclusions: our dence that random peptide library represents a useful tool for the identification tigenic epitope (s) in autoimmune diseases and suggest the existence of epitopes within the intracellular C-terminal domain of IA-2 protein, with the aminoacids representing possibly the main one.

S 15
imal Models for Type 1
d Type 2 Diabetes

SERVATION OF C-PEPTIDE AND INDUCTION OF TH2 TOKINES IN TYPE 1 DIABETES NEWLY DIAGNOSED ULTS VACCINATED WITH DIAPEP277

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ground and Aims: The long-term preservation of the endogenous production could have significant clinical benefit for type 1 diabetes as it could lead to improved metabolic control and decreased ary complications. The aim of this clinical study was to test the y of DiaPep277; a peptide vaccine derived from hsp60 that can t diabetes in the NOD mouse.

ds, Design and follow-up: The study was single-blind, randomized and e-controlled. DiaPep277 was administered 4 times at 1 mg s.c. dose. ell function was evaluated by glucagon-stimulated C-peptide before er each vaccination timepoint. The immune responses to the vaccine e tested by determining the T-cell cytokine profile by ELISPOT analysis. s: After 3 vaccinations with DiaPep277, administered at time 0, 1 and ths, the stimulated C-peptide level of the DiaPep277 vaccinated was significantly increased to 165%±20 over basal secretion, ared to 48%±15 (mean±SD, p=0.048). The effect of the vaccination ed in the immunological parameters as well, as determined by the profiles. Following the 1st and 2nd vaccination with DiaPep277, a increase in Th2 cytokines was evident. The frequency of T cells ing IL-5, IL-10 and IL-13 after in vitro stimulation with either hsp60 or 277 was elevated in the vaccinated group compared to their ive pre-vaccination responses. Responses to bacterial antigens were ed.

sions: DiaPep277 vaccination administered to newly diagnosed type tics patients induced a specific shift to Th2 responses. Vaccinated s maintained statistically significant higher beta-cell function. These ary clinical results are highly encouraging for the therapeutic l of DiaPep277 in type 1 diabetes.

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PENTOXIFYLLINE INDUCED MODULATION OF DIABETES DEVELOPMENT IN BB RATS; A ROLE FOR IL-10 AND TNF-α?

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Background and Aims: Diabetes prone (DP) BB rats spontaneously develop diabetes between 60 and 100 days of age. Diabetes resistant (DR) BB rats can be induced to develop diabetes in 30 days by poly IC and anti-RT6. The phospho-diesterase inhibitor Pentoxifyllins (PTX) has been demonstrated to reduce the development of diabetes in NOD mice and the induction of EAE. PTX suppresses TNF-α and enhances IL-10 production *in vitro*. Here, we studied the effect of PTX on the development of diabetes in both BB rat models of IDDM and we investigated whether these effects might be related to modulation of TNF-α and IL-10.
Materials and Methods: DP-BB rats recieved PTX mixed with their food from weaning until day 130, from weaning until day 60 and from day 60 to day 130. The uptake of PTX was approximately 80mg/kg body-weight. Diabetes was induced in DR-BB rats with low dose poly IC and anti RT6. PTX (80mg/kg body weight) was administered intra peritoneally (i.p.) simultaneously. Another group of DR-BB rats recived only PTX i.p. for 7 days. At the end of this treatment, blood was taken and whole blood cultures were performed to establish cytokine production.
Results: PTX delayed the onset of diabetes and reduced the incidence of the disease (P<0.02, Log Rank test, Kaplan Meier survival curves) in the DP-BB rats treated from day 60. In DR-BB rats, PTX treatment resulted in a ten days delay of the onset of diabetes. Injection of DR-BB rats with PTX suppressed TNF-α production by 50%, but enhanced IL-10 production by 30% in whole blood cultures as compared to the placebo treated animals (P<0.05, Mann Whitney U test).
Conclusions: Taken together, these results demonstrate that timing of PTX treatment determines the protective effect of PTX on the onset of diabetes in the DP-BB rat. Since generally, TNF-α is detrimental and IL-10 is protective in the context of diabetes development, the observed PTX-induced suppression of TNF-α and enhancement of IL-10 might be the underlying mechanism for protection or delay.

Diabetologia (1994) 37: 1195–1201

Diabetologia

Prevention of diabetes mellitus in non-obese diabetic mice by Linomide, a novel immunomodulating drug

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Summary Oral administration of the synthetic immunomodulating drug quinoline-3-carboxamide (Linomide) in the drinking water to 5-week-old female non-obese diabetic (NOD) mice resulted in complete protection from insulitis and maintenance of normal glucose tolerance for over 40 weeks (impaired glucose tolerance: treated $n = 2$ of 18; control $n = 17$ of 18, $p < 0.0001$). Delayed administration of the drug at 16 weeks resulted in slowing of the progression to diabetes when assessed at 42 weeks (treated with diabetes $n = 7$ of 25; control with diabetes 25 of 43, $p < 0.0234$). No gross changes of immune system cell phenotype or function were observed in the Linomide-treated group. Adoptive transfer of spleen and lymph node cells from treated female NOD mice into sub-lethally irradiated male recipients failed to transfer diabetes, whereas a similar transfer of cells obtained from untreated age-matched controls resulted in diabetes in all secondary recipients (diabetes in control group $n = 12$ of 13; in Linomide group $n = 0$ of 11, $p < 0.0001$). Linomide pretreatment of the secondary recipients also inhibited the transfer of diabetes (diabetes in pretreated group $n = 2$ of 9, control group $n = 12$ of 13, $p < 0.015$), as did adoptive co-transfer of cell mixtures obtained from treated female NOD mice, free of diabetes, and from diabetic NOD female mice (diabetes in Linomide group $n = 4$ of 9; in control group 7 of 7, $p < 0.0337$). Our data indicate that Linomide-treated NOD mice generate immune cells with the capacity to downregulate responses to beta-cell antigens, apparently through immunoregulation rather then antigen non-specific immunosuppression. Based on our findings and considering the lack of severe side effects of orally administered Linomide in man, this new compound should be considered as a potential drug for treatment of insulin-dependent diabetes mellitus. [Diabetologia (1994) 37: 1195–1201]

Key words Autoimmunity, immunomodulation, insulin-dependent diabetes mellitus, non-obese diabetic mouse.

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Abbreviations: IDDM, Insulin-dependent diabetes mellitus; NK, natural killer; IL, interleukin; NOD, non-obese diabetic; IPGTT, intraperitoneal glucose tolerance test; FITC, fluorescein-5-isothiocyanate; DTAF, 5(4,6 dichlorotriazinyl) aminofluorescein; FACS, fluorescein activated cell sorting; PHA, phythaemagglutinin; Con A, concanavalin A; SEA, staphylococcal extract A; SEB, staphylococcal extract B; SRBC, sheep erythrocytes; HBSS, Hanks buffered salt solution; LPS, lipopolysaccharide; MLR, mixed lymphocyte reaction; DTH, delayed-type, hypersensitivity; BCG, Bacillus Calmette Guerrin; APC, antigen presenting cell; TNF-α, tumor necrosis factor-α.

Insulin-dependent diabetes mellitus (IDDM) is characterized by immune-mediated progressive destruction of the pancreatic beta cells by auto-reacting lymphocytes. An obvious therapeutic approach would be intervention directed against the autoimmune process, with reinduction of tolerance towards self antigens at an early stage of the disease, aimed at preserving a critical mass of pancreatic beta cells necessary for maintenance of normal glucose tolerance.

Linomide (quinoline-3-carboxamide, Roquinimex, LS 2616), is a novel, orally absorbed, immunomodulatory drug that increases the number and function of activated natural killer (NK)-cells [1, 2] enhances delayed type hypersensitivity reactions [3] and increases lymphocyte proliferation in response to T-cell-dependent mitogens accompanied by an enhanced production of interleukin-2 (IL-2) [4]. Moreover, Linomide has been shown to have a potent ameliorative effect in a variety of murine models of human autoimmune disease such as the systemic lupus erythematosus-like syndrome [5, 6] type II collagen induced arthritis [7], and experimental autoimmune encephalomyelitis [8]. In this report, we studied the effect of long-term oral administration of Linomide on the course of insulitis and diabetes in the non-obese-diabetic (NOD) mouse, a well-characterized mouse strain that serves as a model for human IDDM.

Materials and methods

Mice. Male and female NOD mice were obtained from Bomholtgård (Laven, Denmark) and BALB/c mice were bred locally. All mice were maintained in a regular animal facility at 21 °C with acidified water (pH 2.7) and mouse chow ad libitum.

Experimental conditions. Linomide (supplied by Pharmacia, Lund, Sweden) was administered at a dose of 0.5 mg/ml in drinking water to five or 16-week-old female NOD mice, the control groups received regular water. With an average (± SD) initial fluid intake of 2.9 ± 0.3 ml/day that was maintained throughout the experiments, the Linomide-treated mice received an initial drug dosage of 82.7 ± 7.7 mg · kg^{-1} · day^{-1} that declined (coincident to weight gain) to 58.3 ± 11.8 mg · kg^{-1} · day^{-1} at 40 weeks of age. Subsequent experimental groups received Linomide at a lower dosage (0.1 and 0.02 mg/ml) and for limited periods: 5 weeks (5 to 10 weeks of age) and 15 weeks (5 to 20 weeks). The level of urine glucose (Labstix; Bayer Diagnostics, Puteaux-Cedex, France), weight and fluid intake were determined on a bi-weekly basis. The onset of diabetes was defined after the appearance of glucosuria on two consecutive determinations. In the initial experiments representative animals were killed at 12 weeks of age for histopathological examination; spleen and lymph node cells were harvested for characterization of mononuclear cell phenotype, in vitro proliferative responses and cytolytic activity. At age 16 weeks an intraperitoneal glucose tolerance test (IPGTT) was performed as follows: blood was drawn from the paraorbital plexus at 0 min and 60 min after the i.p. injection of glucose, 1 g/kg body weight. Plasma glucose levels were determined with a Glucose Analyzer 2 (Beckman Instruments, Fullerton, Calif., USA). At 40 weeks of age an additional IPGTT was done and the following day animals were killed for histopathological evaluation and for various assays as described for age 12 weeks.

Histopathology. Pancreatic tissue was fixed in 10 % formalin, embedded in paraffin and thin sections were stained with haematoxylin and eosin. Sections containing a total of 25 islets from each pancreas were reviewed and scored on a blind basis according to the method of Charlton et al. [9]. Briefly, 25 islets from each pancreas were scored according to the following grading system: 0- normal islet; 1- peri-insulitis < 25 % of circumference; 2- peri-insulitis > 25 % of circumference; 3- insulitis penetrating the islet; 4- pan-insulitis with no evidence of normal islet cells. The sum of scores of each pancreas is expressed as a percentage: 0 %- normal islets, 100 %- pan-insulitis involving all islets. Other internal organs were examined for signs of drug toxicity.

Mononuclear cell phenotype characterization. Cell surface phenotype of spleen and lymph node cells obtained from control (untreated) and Linomide treated (0.5 mg/ml in drinking water) female NOD mice was determined as follows: Aliquots of 2×10^6 cells were incubated with each of the following antibodies: fluorescein activated cell sorting (FITC) conjugated anti-mouse CD3-ε monoclonal antibody, FITC conjugated rat anti-mouse L3T4 (CD4) monoclonal antibody, R-phycoerythrin conjugated rat anti-mouse Lyt-2 (CD8a) monoclonal antibody, phycoerythrin conjugated mouse anti-mouse NK-cell specific monoclonal antibody (all purchased from Pharmingen, San Diego, Calif., USA), fluorescein (DTAF) conjugated affinipure f(ab)' 2 fragment goat anti-mouse IgG, f(ab)' 2 fragment specific (Jackson Immunoresearch Laboratories Inc., Baltimore, Md., USA), rat monoclonal antibody to murine type 3 complement receptor MCA 711 (Serotec, Oxford, UK). Cells were washed with ice-cold phosphate buffered saline containing 1 % sodium azide and all incubations and washes were carried out at 4 °C. Freshly stained cells were analysed by FACS (FACStar plus, Becton-Dickenson, San Jose, Calif., USA). Background fluorescence was determined with cells alone.

Mononuclear cell function assessment. The following in vitro responses of pooled spleen and lymph node cells were determined: proliferative responses to T-cell dependent mitogens: PHA (Wellcome, Beckenham, UK) Con A (Sigma St. Louis, Mo., USA), recombinant human IL-2 (a gift from Dr. C.R. Franks. Eurocetus, the Netherlands), IL-4 (as supernatant of mouse cell lines engineered to constitutively secrete the cytokine [11]) and staphylococcal extract A and B (SEA and SEB) (provided by Pharmacia, Lund, Sweden) as well as to B-cell dependent mitogens: lipopolysaccharide (LPS) (Difco, Detroit, Mich., USA), and alloantigens (one-way mixed lymphocyte reaction (MLR) against irradiated [3000 cGy] C_3H/HeJ cells). Cells were cultured in RPMI 1640 medium supplemented with 10 % heat-inactivated human AB serum in 0.2 ml flat-bottomed microtitre plates containing 4×10^5 cells/well and responses were assessed by ^3H-thymidine uptake as described [10]. IL-2 dependent cytolytic activity was tested against ^{51}Cr-labelled NK sensitive (YAC-1) and NK resistant (p815) target cells [11]. Inhibition of T-cell dependent responses to phythaemagglutinin (PHA) or allogeneic lymphocytes (C3H/HeJ) was assayed by adding irradiated and non-irradiated NOD lymphocytes in mixed lymphocyte co-cultures [12].

Adoptive transfer. In preliminary calibration experiments it was possible to adoptively transfer diabetes to sublethally irradiated (550 cGy) male recipients with 10, 20, and 30×10^6 cells, therefore this range of inocula has full potential for induction of the disease. Twenty to 30×10^6 spleen and lymph node cells were obtained from 30-week-old diabetic or age-matched Linomide treated (0.5 mg/ml) female NOD mice and injected i.v. into male recipient mice 1 day after subletbal irradiation whole body (550 cGy). Another group of male recipients from female diabetic donors were either pretreated with Linomide (0.5 mg/ml) for 8 weeks or put on the drug after the procedure. In co-transfer experiments, 20×10^6 cells from diabetic mice were mixed with either 20×10^6 or 40×10^6 cells from

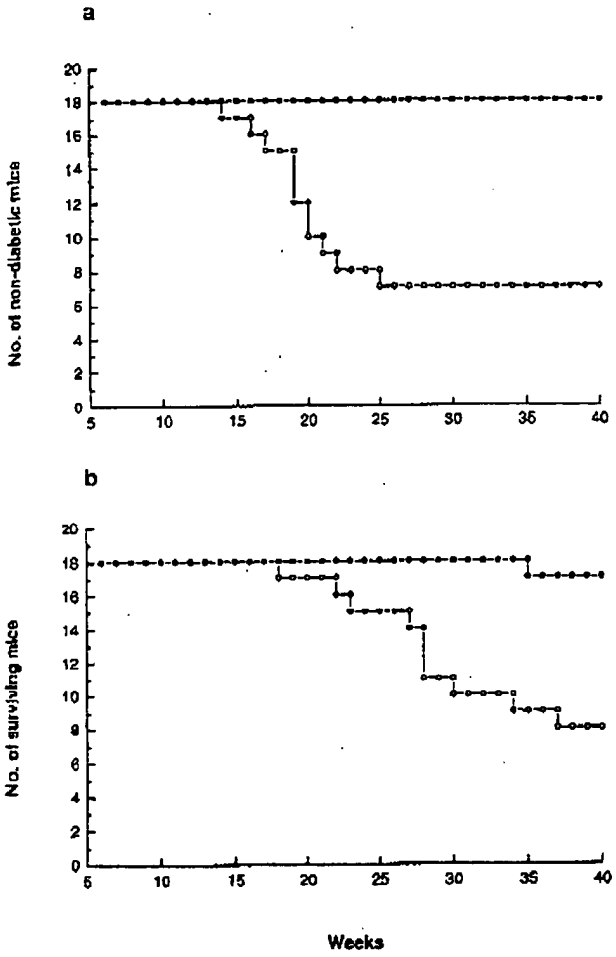

Fig. 1a, b Cumulative incidence of diabetes (a) and mortality (b) in female NOD control (○) and Linomide-treated mice (0.5 mg/ml in drinking water starting from week 5, ●) followed for 40 weeks. The differences between the two groups were highly significant (diabetes $p < 0.0001$; mortality $p < 0.0027$). The presence of diabetes was ascertained on the basis of at least two consecutive determinations of glucosuria greater than 111 mmol/l. The single death in the treatment group was not due to diabetes

Linomide-treated donors. T cells from diabetic mice were prepared by pre-adhering the cell suspension on plastic dishes for 2 h at room temperature and passage of non-adherent cells through a nylon wool column.

Delayed-type hypersensitivity responses. DTH responses to sheep erythrocytes (SRBC) were determined 1 week after immunization with 5×10^5 SRBC HBSS injected i.v. to diabetic and age-matched Linomide-treated female NOD mice. The left anterior footpad was injected with 5×10^8 SRBC in NaCl and the contralateral foot pad was injected with NaCl alone. After 24 h footpad thickness was measured with a micrometer and regional lymph node cell proliferative responses were determined as previously described [13]. Net responses were derived by subtracting control (NaCl) values from SRBC values and expressed in mm and ^{3}H-thymidine incorporation in cpm for the two parameters, respectively.

Statistical analysis

Student's two tailed *t*-test was used for the comparison of means of experimental groups; Fisher's exact test was used for contingency table analyses.

Results

Occurrence of diabetes. Groups of female NOD mice were treated with Linomide 0.5 mg/ml in drinking water starting at 5 weeks of age. Figure 1a shows the cumulative appearance of diabetes in treated mice in comparison with untreated controls. As expected, at approximately 15 weeks of age, untreated animals started to develop diabetes, reaching a cumulative incidence of 61 % at 40 weeks. In sharp contrast, the Linomide-treated group remained free of diabetes throughout the duration of the experiment. At the end of these experiments, there was a significant decrease in the weight of the mice in the treated groups (treated 22.7 ± 2.6 g; control 25.9 ± 2.8 g, $p < 0.0006$); however, they appeared well and remained alive (Fig. 1b). Detailed histological examination of skin, brain, heart, lungs, liver, kidneys, and small and large bowel revealed no evidence of drug toxicity. In order to obtain better assessment of metabolic control, IPGTTs were done at 16 weeks (data not shown) and 40 weeks of age (Fig. 2): with the exception of two mice, the Linomide-treated group demonstrated a normal tolerance test indistinguishable from normal control BALB/c mice, whereas the vast majority of the surviving untreated mice were either diabetic or severely glucose intolerant (Fig. 2).

Effect of Linomide dosage and duration of treatment. In subsequent experiments, we assessed the effect of Linomide dosage, duration of treatment and delayed drug administration on the incidence of diabetes: Linomide, given at either a lower dosage (0.1 and 0.02 mg/ml) or at a dose of 0.5 mg/ml for limited periods of 5 or 15 weeks at the onset (5 weeks of age), resulted in protection from diabetes (Table 1). In order to assess whether Linomide was effective after the onset of insulitis, we assessed the incidence of diabetes in mice receiving the drug only from 16 weeks of age (as expected, no glucosuria was noted at this age). This regimen resulted in a marked delay in the appearance of the disease, with a cumulative incidence after 42 weeks of 28 % in comparison to 58 % in the control group (Table 1).

Pancreatic histology. Histological examination of the pancreas (done in a blinded manner by E.R.) at 12 and 40 weeks of age revealed a paucity of islets, the surviving islets being afflicted with typical insulitis in all untreated mice. In the two Linomide-treated, glucose intolerant mice (Fig. 2), both peri-insulitis in

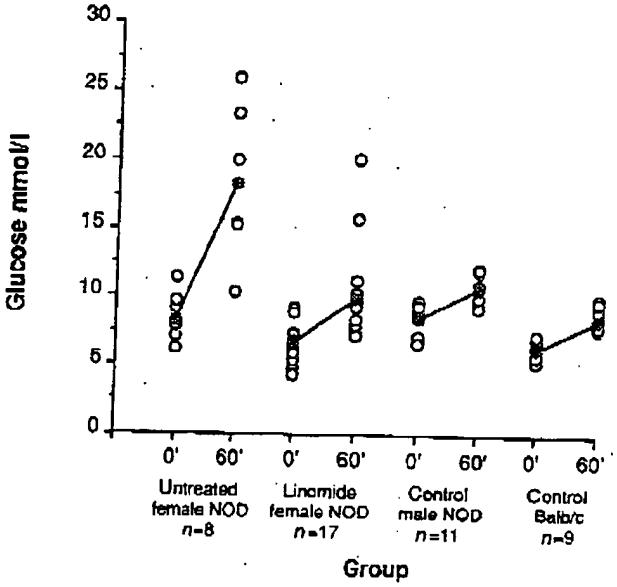

Fig. 2. Intraperitoneal glucose tolerance test (1 g/kg body weight glucose given before and 60 min after blood sampling from the orbital capillary plexus) done in surviving untreated control female NOD mice at 40 weeks, Linomide-treated female NOD mice at 40 weeks (0.5 mg/ml in drinking water from week 5), untreated male NOD control mice (5 weeks of age) and control BALB/c mice. The basal glucose values were significantly elevated in the untreated female NOD controls (untreated vs Balb/c $p < 0.013$), but not in the Linomide-treated group (Linomide vs Balb/c- N.S.). The mean 60-min glucose values were the same for the Linomide-treated females, male NOD and Balb/c groups and significantly higher in the untreated female NOD controls (untreated vs male NOD, $p < 0.012$). Plasma glucose levels were determined by the glucose oxidase method. Lines connecting stippled circles depict mean values

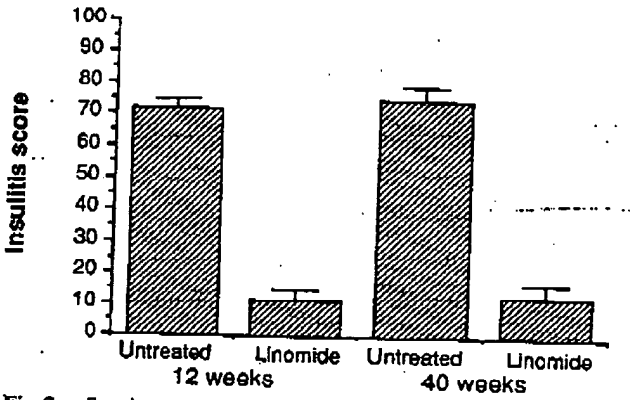

Fig. 3. Semi-quantitative assessment of insulitis severity (score ± SEM) in Linomide-treated (0.5 mg/ml in drinking water from week 5) and untreated control mice at 12 weeks (control $n = 6$; Linomide $n = 6$, $p < 0.001$) and 40 weeks (controls $n = 8$; Linomide $n = 17$, $p < 0.001$). The 12 and 40 week scores of both the control and Linomide-treated groups did not differ significantly

Table 1. The effect of Linomide dosage, duration of treatme and timing of treatment and on the cumulative incidence diabetes in female NOD mice at 42 weeks of age

Linomide dosage	Incidence and (%) of diabetes at 42 weeks	p value
Control (untreated):	25/43 (58)	–
0.5 mg/ml (weeks 5–42)	0/18 (0)	< 0.000
0.1 mg/ml (weeks 5–42)	0/17 (0)	< 0.000
0.02 mg/ml (weeks 5–42)	1/20 (5)	< 0.000
0.5 mg/ml (weeks 5–10)	2/10 (20)	< 0.039
0.5 mg/ml (weeks 5–20)	2/10 (20)	< 0.039
0.5 mg/ml (weks 16–42)	7/25 (28)	< 0.023

[a] Compared to control group, Fisher's exact test
Each row depicts the summary of an individual experiment excluding the control data that was pooled from three experiments. Values in parentheses denote the duration and timing of drug administration

some islets and pan-insulitis in others were observed. However, most treated animals showed islets predominantly normal in size, number and morphology: there was only occasional mild peri-insulitis in a minority of islets, reflected by the semi-quantitative insulitis score shown in Fig. 3.

Spleen and lymph node mononuclear cell phenotype. We compared the phenotype of spleen and lymph node cells obtained at 40 weeks of age from Linomide-treated and age-matched untreated controls. FACS analysis showed no significant difference in the expression of cell surface CD3, CD4, CD8, B-cells, MAC-1 cells and NK cells (Table 2).

In vitro cell mediated proliferative responses. Functional in vitro studies of cell-mediated proliferative responses assessed by ^{3}H-thymidine uptake in response to T-cell dependent mitogens (PHA, Con A, IL-2, IL-4, SEA and SEB), B-cell dependent mitogen (LPS) and MLR showed no differences between Linomide-treated and control NOD mice (Table 3 and data not shown). Likewise, IL-2-induced cytolytic responses against ^{51}Cr-labelled NK sensitive (YAC-1) and NK resistant (p815) target cells at 40 weeks of age revealed no significant differences between Linomide-treated mice (0.5 mg/ml and 2.5 mg/ml) in comparison with untreated age-matched controls (data not shown). Irradiated and non-irradiated splenocytes obtained from Linomide-treated NOD mice did not block T-cell dependent proliferative responses to either PHA or allogeneic lymphocytes (C3H/HeJ) in mixed lymphocyte co-cultures when mixed in equal proportions to the responding effector cells (data not shown).

Delayed type hypersensitivity (DTH) responses. DTH responses to SRBC were determined in untreated (control) and Linomide-treated (0.5 mg/ml) female NOD mice. Both groups showed a wide inter-subject

Table 2. Representative experiment of cell surface phenotype of spleen cell and lymph node cells obtained from control (untreated) and Linomide-treated (0.5 mg/ml in drinking water) female NOD mice at 40 weeks of age. The present and subsequent experiments (data not shown), showed no significant trends

	CD3	CD4	CD8	B-cells	Mac-1	NK cells
Untreated:	20.4 ± 2.8	10.3 ± 4.2	7.6 ± 1.2	27.1 ± 4.5	4.4 ± 0.6	3.3 ± 0.3
Linomide:	25.2 ± 4.1	17.4 ± 2.5	8.7 ± 1.5	30.9 ± 2.2	2.9 ± 0.5	2.1 ± 0.3

Data presented as mean ± SD

Table 3. In vitro proliferative responses of spleen and lymph node cells to mitogens in control and Linomide-treated (0.5 mg/ml in drinking water) female NOD mice

	^{3}H thymidine uptake (cpm ± SD)[a]			
	Untreated		Linomide-treated	
Stimulating agent	Basal	Stimulated	Basal	Stimulated
PHA (10 μg/ml)[b]	7,837 ± 734	129, 408 ± 7,524	9,259 ± 1,757	142,156 ± 5,209
Con A (10μg/ml)[b]	"	212,514 ± 24,733	"	195,167 ± 19,042
SEA (1 μg/ml)[b]	"	61,482 ± 7,817	"	79,314 ± 11,402
SEB (1 μg/ml)[b]	"	16,121 ± 1,017	"	32,896 ± 7,513
rhIL-2 (100 μg/ml)[c]	538 ± 127	2,373 ± 1,376	672 ± 148	1,846 ± 463
IL-4 supernatant (0.2 ml)[c]	"	13,482 ± 263	"	15,193 ± 813
LPS (50 μg/ml)[c]	"	23,033 ± 4,324	"	18,078 ± 3,570

[a] Representative experiments (of two or three experiments) on cell samples pooled from three animals from each treatment group. There were no significant differences between the untreated and treated groups. Additional data obtained at 16 weeks of age again did not demonstrate any differences of mitogen responses between the two groups (data not shown). [b] After 1 week of treatment. [c] Determined at the end of the experiments at 40 weeks of age

variation. Net footpad thickness was 0.38 ± 0.16 mm in controls vs 0.48 ± 0.14 mm in the Linomide-treated group. ^{3}H-thymidine incorporation in the lymphocyte proliferation assay was 305 ± 332 cpm (mean ± SEM) in controls (n = 8) vs 335 ± 221 cpm in Linomide-treated mice (n = 9). There were no significant differences between the two groups.

Adoptive transfer of diabetes. Mixtures of spleen and lymph node cells or purified T cells obtained from diabetic mice injected i.v. into sub-lethally irradiated male NOD recipients resulted in diabetes appearing between days 20 to 60 approximately, with a cumulative incidence of 92 % at 100 days (Table 4, groups A-C). In contrast, a similar adoptive transfer of cells obtained from normoglycaemic, Linomide-treated female NOD mice did not result in any evidence of diabetes (as determined by glucosuria) during the same observation period (Table 4, group D). Adoptive transfer of spleen and lymph node cell mixtures obtained from a pool of untreated, diabetic female NOD mice into male recipients that were placed on Linomide therapy starting from the date of cell transfer did not confer protection (Table 4, group E and F). In contrast, male recipients pretreated with Linomide for 10 weeks prior to cell transfer were protected from diabetes as compared with untreated male recipient controls that received similar inocula (Table 4, group G). Adoptive co-transfer of 20×10^6 cells from diabetic and Linomide-treated female mice at a 1:1 ratio did not alter the course of the disease (Table 4, group H). However, when the inoculum from the Linomide-treated mice was increased to 40×10^6 cells, (2:1 ratio, Linomide vs diabetic) diabetes was suppressed in more than 50 % of the animals, in comparison to 100 % in the control group (Table 4, groups I vs J).

Discussion

The data presented suggest that in the NOD mouse, prevention of autoimmune insulitis and diabetes can be accomplished by oral administration of Linomide without significant side effects. Although the details of the mechanism of Linomide action on the autoimmune process are yet unknown, sustained antigen non-specific immunosuppressive effects were ruled out by comparable cell surface phenotype, in vitro responses of mononuclear cells to T- and B-cell dependent mitogens and mixed lymphocyte reaction, cytotoxicity assays against NK-sensitive and NK-resistant target cells as well as the in vivo DTH responses in treated and control mice. Establishment of unresponsiveness to self antigens by Linomide, as confirmed by the adoptive transfer experiments, in the presence of normal or even enhanced T-cell dependent mitogenic responses, suggests that Linomide may be effective for immunoregulation of self-reactive lymphocytes. The inhibition of diabetes in Linomide-pretreated secondary adoptive recipients of spleen and lymph node cells derived from diabetic

Table 4. Effect of adoptive transfer of spleen and lymph node cells on the incidence of diabetes in sublethally (550 cGy) irradiated 2-month-old male NOD recipients

Group No.	Source of lymph node and spleen cells	Cell inoculum size	Number of recipients	Linomide therapy	Number of diabetic mice (%) at 90 days post-transfer	*p* value[e]
A	Diabetic[a]	20×10^6	13	–	12(92)	–
B	Diabetic	30×10^6	5	–	5(100)	–
C	T cells from diabetic	20×10^6	6	–	6(100)	–
D	Linomide treated	20×10^6	11	–	0(0)	< 0.0001
E	Diabetic	20×10^6	5	+[b]	5(100)	NS
F	Diabetic	30×10^6	6	+[b]	6(100)	NS
G	Diabetic	20×10^6	9	++[c]	2(22)	< 0.0015
H	*Co-transfer* Diabetic Linomide treated	20×10^6 20×10^6	7	–	6(86)	NS
I	Diabetic Linomide treated	20×10^6 40×10^6	9	–	4(44)	< 0.0337
J	Diabetic Non-diabetic	20×10^6 40×10^6	7	–	7(100)	–

[a] Aliquots of $20–30 \times 10^6$ cells were obtained from 30-week-old diabetic or age-matched Linomide-treated (0.5 mg/ml) female NOD mice and injected i.v. into male recipient mice 1 day after irradiation. [b] Adoptive male recipients were put on Linomide (0.5 mg/ml) starting one day after the cell transfer. [c] Adoptive male recipients were treated with the Linomide (0.5 mg/ml) for 10 weeks prior to cell transfer [d] Comparison of groups D, E, F and G to group A; groups H and I to group J. Fisher's exact test. Diabetes was ascertained on the basis of two consecutive determinations of glucosuria. In adoptive recipients of cells from diabetic donors, glucosuria usually appeared between days 20–60 post cell transfer; the cumulative incidence of diabetes was assessed 90 days after the procedure

donors, in recipients receiving cell mixtures from both diabetic and Linomide-treated donors and the activity of the drug when given after the onset of insulitis all indicate that Linomide presumably generates immune cells capable of active suppression of the beta-cell directed immune response.

Treatment of NOD mice with various agents associated with macrophage activation, such as Bacillus Calmette Guerrin (BCG) [14] and complete Freund's adjuvant [15–18] result in marked amelioration of diabetes. Conversely, it has recently been shown that defects of macrophage differentiation and function are present in NOD mice [19]. Linomide has been found to have a profound immunostimulatory effect on macrophages of BALB/c mice [4] and in preliminary experiments we have observed an increase in the Mac-3/Mac-1 expression ratio on NOD peritoneal cells obtained from mice treated with the drug, indicating a possible effect on activation and maturation of macrophage phenotype (unpublished observations). Thus, Linomide might prevent generation of anti-"self" responses by either enhancing APC-mediated tolerogenic responses, or alternatively by down regulating anti-"self" responses through macrophage dependent suppression signals, a phenomenon that was previously described as "suppressor macrophage" activity [14].

TNF-α has disparate effects on beta-cell function that are dependent on the mode of administration. On th one hand, TNF-α adversely modifies adhesion properties of pancreatic beta cells [20] and potentiates the in vitro beta-cell cytotoxic effect of IL-1 [21] and interferon-γ [22, 23]. On the other hand, systemic administration of TNF-α to NOD mice results in marked amelioration of diabetes [24]. Recently, it has been shown that TNF-α is produced selectively by pancreatic beta cells in response to exposure to IL-1β [25], thus possibly contributing to the selective destruction of beta cells in IDDM. Since Linomide has a marked inhibitory effect on exotoxin-induced TNF-α production in mice [26], an additional mode of drug action might be suppression of endogenous production of TNF-α by NOD beta cells. This possibility is currently under investigation in our laboratory.

In view of the fact that the beta-cell antigen(s) involved in initiation of the autoimmune response are unknown, direct in vitro documentation of antigen-specific anergy is currently not feasible. However, in experiments done in parallel in experimental autoimmune encephalomyelitis (EAE) in SJL/J mice we have recently documented Linomide-induced unresponsiveness to myelin basic protein, the antigen responsible for induction of the disease, while antigen non-specific T-cell responses to Con A and PHA, as in Linomide-treated NOD mice, were not affected [8]. Taken together, our data suggest that Linomide therapy results in re-induction of tolerance to beta-cell components that serve as immunogens for autoreactive cells, thus preventing or ameliorating autoimmune insulitis and diabetes, depending on the

stage of disease at which therapy was instituted. Regardless of the mechanism involved, once unresponsiveness was established, lymphocytes obtained from Linomide-treated normoglycaemic donors failed to transfer the disease by adoptive transfer experiments.

From our studies, it is evident that complete protection of female NOD mice from diabetes for over 42 weeks can be conferred by a limited period of treatment at the onset of the disease (weeks 5 to 10), without the decrease in body weight observed in the chronically-treated animals (data not shown). Moreover, treatment at a dose of 0.5 mg/ml within this brief therapeutic window is more protective than chronic treatment with lower doses of the drug for the duration: IPGTTs performed at 42 weeks in the various dosage groups (Table 1), revealed a progressive impairment of glucose tolerance with reduction of drug dosage, whereas glucose tolerance in the limited treatment group was normal and comparable to that of the chronic 0.5 mg/ml group (data not shown).

Linomide, which is orally absorbed and devoid of any major side effects in experimental animals and man [27], might be a useful drug for treatment of IDDM at early stages of the disease, because it apparently suppresses autoreactivity, even in NOD mice with established insulitis, without causing gross immunosuppression.

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ANTI-CD3 MONOCLONAL ANTIBODY IN NEW-ONSET TYPE 1 DIABETES MELLITUS

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ABSTRACT

Background Type 1 diabetes mellitus is a chronic autoimmune disease caused by the pathogenic action of T lymphocytes on insulin-producing beta cells. Previous clinical studies have shown that continuous immune suppression temporarily slows the loss of insulin production. Preclinical studies suggested that a monoclonal antibody against CD3 could reverse hyperglycemia at presentation and induce tolerance to recurrent disease.

Methods We studied the effects of a nonactivating humanized monoclonal antibody against CD3 — hOKT3γ1(Ala-Ala) — on the loss of insulin production in patients with type 1 diabetes mellitus. Within 6 weeks after diagnosis, 24 patients were randomly assigned to receive either a single 14-day course of treatment with the monoclonal antibody or no antibody and were studied during the first year of disease.

Results Treatment with the monoclonal antibody maintained or improved insulin production after one year in 9 of the 12 patients in the treatment group, whereas only 2 of the 12 controls had a sustained response (P=0.01). The treatment effect on insulin responses lasted for at least 12 months after diagnosis. Glycosylated hemoglobin levels and insulin doses were also reduced in the monoclonal-antibody group. No severe side effects occurred, and the most common side effects were fever, rash, and anemia. Clinical responses were associated with a change in the ratio of CD4+ T cells to CD8+ T cells 30 and 90 days after treatment.

Conclusions Treatment with hOKT3γ1(Ala-Ala) mitigates the deterioration in insulin production and improves metabolic control during the first year of type 1 diabetes mellitus in the majority of patients. The mechanism of action of the anti-CD3 monoclonal antibody may involve direct effects on pathogenic T cells, the induction of populations of regulatory cells, or both. (N Engl J Med 2002;346:1692-8)

TYPE 1 diabetes mellitus is a T-cell–mediated autoimmune disease that begins, in many cases, three to five years before the onset of clinical symptoms, continues after diagnosis, and can recur after islet transplantation.[1-3] The effector mechanisms responsible for the destruction of beta cells involve cytotoxic T cells as well as soluble T-cell products, such as interferon-γ, tumor necrosis factor α, and others.[4] Such observations have led to clinical trials with immunomodulatory drugs such as cyclosporine, azathioprine, prednisone, and antithymocyte globulin, which were shown to cause transient improvement in clinical measures and to enhance the rate of non–insulin-requiring remissions when initiated soon after diagnosis.[5-8] Unfortunately, the toxic effects of such drugs, concern about the risk associated with immune suppression, and the need for continuous treatment in an otherwise healthy, young population limit the use of these agents.[9]

We,[10] as well as Chatenoud et al.,[11-13] have reported that treatment of mice with a modified monoclonal antibody against CD3 that had been altered to prevent binding to the Fc receptor prevents or reverses diabetes in nonobese diabetic mice and other mouse models of type 1 diabetes mellitus. This antibody can be used without toxic effects such as the high fevers and hypotension that are typically associated with T-cell activation in vivo.[10-13] Initial studies in which a humanized anti-CD3 molecule — that is, a monoclonal antibody called hOKT3γ1(Ala-Ala) that contains the binding region of OKT3 but a mutated Fc region that prevents it from binding to the Fc receptor — was used in patients with renal-allograft rejection demonstrated efficacy similar to that of OKT3 with markedly fewer side effects.[14,15] On the basis of these observations, we initiated a randomized, controlled, phase 1–2 trial of this agent in patients with new-onset type 1 diabetes mellitus. In this report, we describe the results among patients who were followed for one year after treatment.

METHODS

Study Patients

Patients between 7½ and 30 years of age in whom type 1 diabetes mellitus had been diagnosed within the previous six weeks (or who had been discharged from the hospital within that period after receiving such a diagnosis) were eligible for participation. All

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patients had one or more of the following types of antibodies: anti-GAD (glutamic acid decarboxylase), anti–islet-cell antibody 512 (ICA512), and anti-insulin antibody. Patients were treated by their personal physicians, received at least three injections of short-acting or intermediate-acting insulin, and did not discontinue insulin therapy during the study period. The study was approved by the institutional review boards at Columbia Presbyterian Medical Center, the National Institute of Diabetes and Digestive and Kidney Diseases, the University of Utah, and the University of California at San Francisco. All patients or their parents provided written informed consent, and written assent was obtained from minor subjects.

Study Protocol

The data reported here were obtained between May 1999 and August 2001. Eligible patients were randomly assigned to the control group or the monoclonal-antibody group. Patients in the control group underwent metabolic and immunologic studies but did not receive monoclonal antibody and were not hospitalized. Blood samples were drawn for immunologic studies and measurement of glycosylated hemoglobin when the patient entered the study, and a four-hour mixed-meal tolerance test was performed after the morning dose of insulin and the previous evening's dose of long-acting insulin had been withheld.[7]

Nine patients in the monoclonal-antibody group were hospitalized, and the other three received monoclonal antibody on an outpatient basis. All 12 patients received a 14-day course of the anti-CD3 monoclonal antibody hOKT3γ1(Ala-Ala) administered intravenously (1.42 μg per kilogram of body weight on day 1; 5.67 μg per kilogram on day 2; 11.3 μg per kilogram on day 3; 22.6 μg per kilogram on day 4; and 45.4 μg per kilogram on days 5 through 14); the doses were based on those previously used for treatment of transplant rejection.[15] The dosing resulted in median peak and trough serum monoclonal-antibody levels of 133 ng per milliliter (range, 68 to 275) and 51 ng per milliliter (range, 23 to 255), respectively. Flow cytometry was used for the enumeration of CD4+ T cells, CD8+ T cells, and non–T cells and for coating and modulation of the CD3 molecule.[15] Coating of CD3+ cells was maximal (mean [±SD] percentage reduction in fluorescence, 69.2±2.9) by day 12 of monoclonal-antibody treatment. Modulation of the CD3 molecule reached a peak level of 54.0±3.1 percent by day 14.

Patients underwent physical examinations, blood counts, and blood chemistries and were questioned about side effects weekly for two weeks after discharge and every two to three months thereafter. Glycosylated hemoglobin was measured and a mixed-meal tolerance test was performed every six months.

Statistical Analysis

C-peptide levels were measured by radioimmunoassay at the Diabetes Research and Training Center at the University of Chicago.[16] The C-peptide response to the mixed meal was expressed as the total area under the response curve or the incremental area under the curve formed by subtracting the fasting C-peptide level from the response at each time point.[7] A change in the response was considered to have occurred if the response differed by more than 7.5 percent from the response at study entry (7.5 percent being half of the interassay coefficient of variation for the C-peptide assay). Changes in insulin secretion were evaluated by examining the slope of the line described by the three data points (at study entry, 6 months, and 12 months).

Anti-GAD antibody, anti-ICA512, and anti-insulin antibody were measured with radiobinding assays.[17] For genotyping at the HLA-DQA and DQB loci, direct sequencing of exon 2 polymorphisms was used after polymerase-chain-reaction amplification.[18]

Cytokines were measured in serum by enzyme-linked immunosorbent assay (ELISA) (BioSource and Immunotech). Anti-idiotype antibodies were identified by ELISA with the use of plate-bound OKT3 or by flow cytometry to measure the blockade of binding of OKT3 fluorescein isothiocyanate to CD3.[19] Glycosylated hemoglobin levels were measured by latex-agglutination inhibition tests (DCA 2000, Bayer) or by affinity chromatography (Isolab) in the three patients treated at the National Institutes of Health.

Data are expressed as means ±SD. We used repeated-measures analysis of variance to compare the control group and the monoclonal-antibody group in terms of the response to the mixed-meal tolerance test, the glycosylated hemoglobin level, and the required dose of insulin. Comparisons between groups were made with the Mann–Whitney U test. Fisher's exact test was used to assess the effect of monoclonal-antibody treatment on the response to mixed-meal tolerance testing. Statistical analyses were performed with StatView software (SAS Institute).

RESULTS

Enrollment of Study Patients

The average age of patients in the control group was slightly higher than that in the monoclonal-antibody group, but there were no significant differences between the two groups at entry (Table 1). Autoantibodies against at least one type of biochemically defined autoantigen were present in all subjects.

Effects of Antibody Treatment on Circulating Lymphocytes

A transient reduction in the number of circulating lymphocytes occurred with monoclonal-antibody treatment. After the administration of the first full dose of monoclonal antibody on day 5, the absolute lymphocyte count reached a nadir of 26.5±9.0 percent of the base-line lymphocyte count. The changes in the absolute lymphocyte count were due to reductions in the numbers of CD4+ cells, CD8+ cells, and B cells (CD19+ cells) to 36.6±19.0 percent of their pretreatment levels. The reduction in the number of circulating lymphocytes was transient, however, and the number of circulating cells began to rise after the seventh day of treatment. By day 30 (two weeks after the last dose of the monoclonal antibody), the level of circulating lymphocytes reached 123.0±52.0 percent of the pretreatment level.

Release of Cytokines after Treatment

The levels of cytokines were measured in serum after the initial two doses of monoclonal antibody and after the first two full doses on days 5 and 6. Interleukin-6 was detectable in 8 of the patients treated with monoclonal antibody (range of levels, 14 to 225 pg per milliliter), and tumor necrosis factor α was detectable in all 12 patients (range of levels, 7 to 158 pg per milliliter). The circulating levels of these cytokines were maximal after the administration of the second dose of the monoclonal antibody but were considerably lower than levels previously reported in patients with the "cytokine-release syndrome" associated with the administration of OKT3; these levels were consistent with the mild clinical side effects.[13] Interleukin-2 was not detectable in these patients,

TABLE 1. CHARACTERISTICS OF THE PATIENTS AT ENTRY.*

CHARACTERISTIC	MONOCLONAL-ANTIBODY GROUP (N=12)	CONTROL GROUP (N=12)	P VALUE
Sex (no.)			0.64
Male	10	8	
Female	2	4	
Age (yr)			0.15
Median	13	16	
Range	7–27	8–30	
Diabetic ketoacidosis at diagnosis (no.)	3	5	0.67
Glycosylated hemoglobin (%)	9.27±1.59	8.27±1.06	0.14
Fasting C-peptide level (nmol/liter)	0.20±0.13	0.21±0.07	0.77
Anti-GAD65 antibody			
Index	0.48±0.52	0.51±0.63	0.95
No. testing positive	8	9	
Anti-ICA512 antibody			
Index	0.34±0.44	0.35±0.44	0.84
No. testing positive	7	5	
Anti-insulin antibody			
Index	0.76±1.31	0.75±1.31	0.75
No. testing positive	7	8	
HLA-DQ haplotype†			0.40
No. with susceptible alleles	6	9	
No. with resistant alleles	6	3	

*Plus–minus values are means ±SD. GAD denotes glutamic acid decarboxylase, and anti-ICA512 anti–islet-cell antibody 512.

†Diabetes-resistant HLA-DQ (α,β) haplotypes included 0101/0601, 0101/0503, 0102/0602, 0103/0603, 0201/0201, 0201/0303, 0301/0301, and 0501/0301. A haplotype with a resistant allele was designated as a resistant haplotype, whether the other allele was susceptible, neutral, or resistant.

and interferon-γ was detectable in only one patient, whereas interleukin-5 was detected in the serum of nine of the antibody-treated patients (range of levels, 9 to 33 pg per milliliter) and interleukin-10 was detected in the serum of seven patients (range of levels, 5 to 316 pg per milliliter).

Side Effects of Antibody Treatment

Side effects of monoclonal-antibody infusions included mild and moderate fever in 9 of the 12 patients, generally on day 5; mild or moderate anemia in 9 of the 12 (which resolved after day 14); and nausea, vomiting, arthralgia, and headache in 1 patient each. A pruritic urticarial rash developed on the hands and occasionally the trunk and feet of 7 of the 12 patients. The rash appeared after the seventh day of treatment and resolved by day 30. A biopsy of this rash in two patients showed spongiosis consistent with eczematous dermatitis. There was no evidence of vasculitis. Antiidiotype antibodies developed in 6 of the 12 patients within the first month after treatment; but after six months, only 3 patients still had antibodies, and at one year, only 1 had detectable levels. There has been no evidence of long-term toxic effects up to two years after antibody treatment.

Monoclonal-Antibody Treatment and Insulin Production

Antibody treatment significantly reduced the decline in the incremental and total C-peptide responses ($P=0.01$ for both comparisons) (Table 2 and Fig. 1). At the end of one year, the incremental C-peptide response in the monoclonal-antibody group was 109±74 percent of the response to the mixed-meal tolerance test at entry and the total C-peptide response was 103±53 percent of the base-line response, whereas the corresponding values in the control group were 42±35 percent and 49±33 percent of the base-line response. There was an average monthly decrease in the total C-peptide response of 5.52±1.30 nmol per liter per four-hour test in the control group, as compared with an average monthly increase of 0.20±1.86 nmol per liter per four-hour test in the monoclonal-antibody group ($P=0.006$). After one year, seven of the patients in the monoclonal-antibody group had no change or an increase (of more than 7.5 percent) from base line in the incremental response during the mixed-meal tolerance test; the other five had a decrease in the incremental response. By contrast, 11 of the 12 patients in the control group had a decrease in the incremental response ($P=0.03$). Nine of the 12 patients in the monoclonal-antibody group had no change or an increase in the total C-peptide response, whereas 10 of the 12 patients in the control group had a decrease in response ($P=0.01$).

Eleven of the 12 treated patients have been followed for more than 18 months. At 18 months, the mean incremental C-peptide response in these 11 patients was 90±82 percent of the pretreatment level, and the total C-peptide response was 74±39 percent of the base-line level. The incremental response was the same as the base-line response or greater in 6 of the 11 patients, and the total response was the same as the base-line response or greater in 5 of the 11 patients. By contrast, in 9 of the 12 controls studied, the incremental C-peptide response was 35±38 percent of the base-line level ($P=0.07$ for the comparison with the monoclonal-antibody group), and the total C-peptide response was 42±36 percent of the base-line level ($P=0.06$ for the comparison with the monoclonal-antibody group).

Metabolic Control of Diabetes

Antibody treatment resulted in a significant decrease in glycosylated hemoglobin levels ($P=0.008$). At study entry, the average glycosylated hemoglobin level was nonsignificantly higher in the monoclonal-antibody group, but the decline in glycosylated he-

TABLE 2. CHANGES IN THE INCREMENTAL AND TOTAL C-PEPTIDE RESPONSES DURING MIXED-MEAL TOLERANCE TESTING.*

VARIABLE	C-PEPTIDE RESPONSE DURING MIXED-MEAL TOLERANCE TESTING		
	STUDY ENTRY	6 MO	12 MO
	nmol/liter		
Monoclonal-antibody group			
Incremental response	63.1±33.0	69.0±51.2	67.7±62.3
Total response	111.5±50.2	121.1±79.7	114.2±90.6
Control group			
Incremental response	82.7±44.9	50.1±47.4	34.1±30.4
Total response	133.2±50.7	92.6±61.8	66.7±53.0

*Plus–minus values are means ±SD. The total response is the total area under the response curve, and the incremental response is the area under the curve formed by subtracting the fasting C-peptide level from the response at each time point. P=0.01 by repeated-measures analysis of variance for the comparison of the total response in the two groups.

moglobin levels between base line and six months was greater in that group (P=0.01) (Table 3). There were no severe hypoglycemic events in either group.

The improved glycemic control was not due to increased use of insulin in the monoclonal-antibody group. In fact, there was a significant decrease in the use of insulin in the monoclonal-antibody group as compared with the control group (P=0.03) (Table 3). After one year, the average insulin dose in the monoclonal-antibody group was below the level that is considered to indicate clinical remission (0.5 U per kilogram per day).[20] Thus, monoclonal-antibody treatment resulted in improved metabolic control with reduced insulin usage during the first year after the diagnosis of type 1 diabetes mellitus.

Possible Predictors of Clinical Response

There were no differences between the patients with a response to monoclonal-antibody treatment and those with no response in terms of clinical presentation (including the presence or absence of patients with diabetic ketoacidosis), the titers of biochemically defined autoantibodies, the isotype subclasses of the autoantibodies, or the HLA-DQA1 and DQB1 genotypes. The mean fasting C-peptide level at study entry was 0.24±0.13 nmol per liter in subjects who had an increase or no change in the incremental C-peptide response to the mixed-meal tolerance test at six months, as compared with 0.12±0.09 nmol per liter in those who had a decline in the C-peptide response (P=0.13).

The pattern of T-cell repopulation after the nadir in the absolute lymphocyte count correlated with the response to monoclonal antibody. At 3 months (90 days), patients with a response to monoclonal-antibody treatment had a 68 percent increase in the

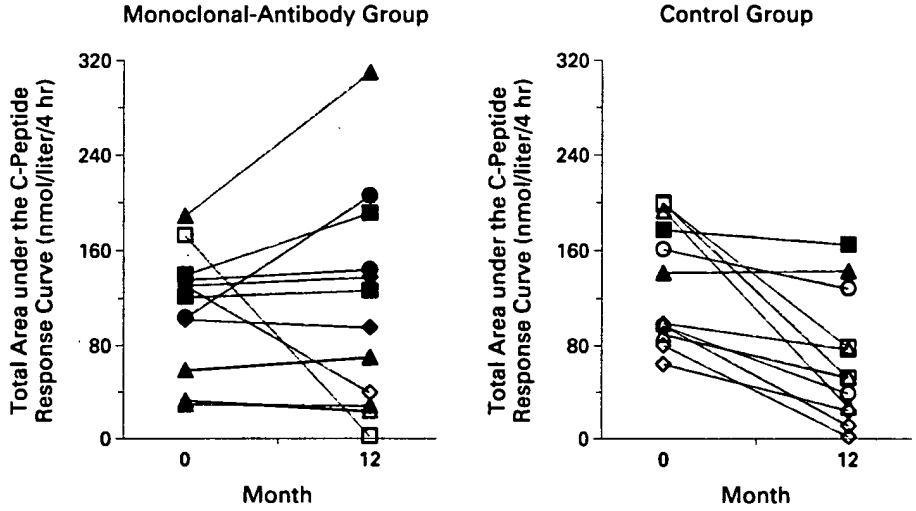

Figure 1. Changes from Study Entry to 12 Months in the Total C-Peptide Response to Mixed-Meal Tolerance Testing.

Data from each control and antibody-treated subject are shown. Solid symbols represent patients who had a sustained or increased C-peptide response, and open symbols represent patients who had a reduced response.

TABLE 3. EFFECTS OF TREATMENT WITH THE MONOCLONAL ANTIBODY hOKT3γ1(Ala-Ala) ON METABOLIC MEASURES.*

MEASURE	STUDY ENTRY	6 MO	12 MO
Glycosylated hemoglobin level (%)†			
Monoclonal-antibody group	9.27±1.59	6.23±0.86	6.98±1.70
Control group	8.20±1.05	7.65±1.41	7.53±1.27
Insulin dose (U/kg of body weight)			
Monoclonal-antibody group	0.57±0.17	0.36±0.26	0.49±0.28
Control group	0.44±0.25	0.52±0.21	0.59±0.17

*Plus–minus values are means ±SD. P=0.008 by repeated-measures analysis of variance for the comparison of the glycosylated hemoglobin level in the two groups, and P=0.03 by repeated-measures analysis of variance for the comparison of the insulin dose in the two groups.

†The normal range is 4.5 to 6.5 percent.

absolute number of repopulating CD8+ T cells, which was reflected in a reduction in the ratio of CD4+ T cells to CD8+ T cells (Fig. 2).

DISCUSSION

Treatment of new-onset type 1 diabetes mellitus with a single course of a monoclonal antibody against CD3 that does not bind to the Fc receptor appears to have arrested the loss of insulin responses during the first year after diagnosis in most, but not all, of the 12 patients we studied. One year after treatment, two thirds of the antibody-treated patients had a C-peptide response to the mixed-meal tolerance test that was the same as or greater than their response at study entry. In contrast, there was a consistent decline in the C-peptide response in 10 of the 12 untreated patients. The decline among control patients is somewhat surprising, since many of these patients entered a clinical "honeymoon" that has been thought to reflect improved insulin secretion after diagnosis. However, our metabolic studies, which used a four-hour provocative test rather than more abbreviated protocols, challenge this notion and suggest that a relentless decline is the natural history of the disease in the majority of patients. At the time of study entry, the control group was slightly older, had lower glycosylated hemoglobin levels, and had greater responses to the mixed-meal tolerance test than the monoclonal-antibody group. These differences between the two groups, although not statistically significant, would tend to bias the results against an effect of the antibody treatment, since patients younger than 18 years of age have generally been found to have more aggressive disease than patients 18 years of age or older.[21,22] Thus, the true antibody effect may have been greater than is apparent from the comparison

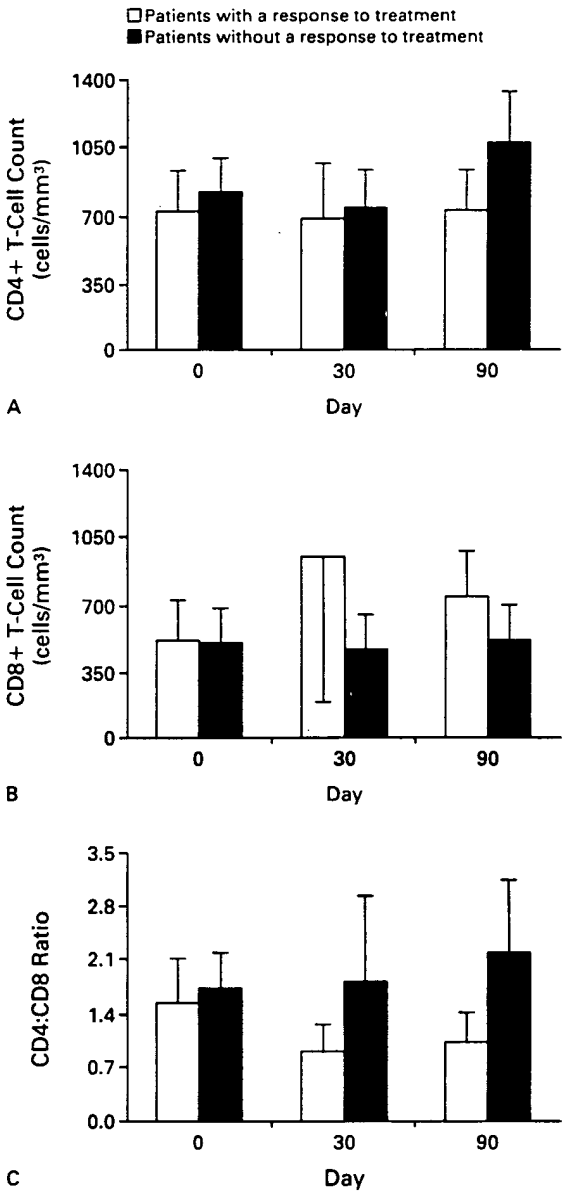

Figure 2. Mean CD4+ and CD8+ T-Cell Counts in the Monoclonal-Antibody Group According to the Presence or Absence of a Response to Treatment.

Panel A shows CD4+ T-cell counts, and Panel B CD8+ T-cell counts. The ratio of CD4+ T cells to CD8+ T cells (Panel C) was reduced in patients who had a clinical response to monoclonal-antibody treatment. The absolute number of each type of T cell was determined by multiplying the percentage of cells by the absolute lymphocyte count. The CD4:CD8 ratio was decreased in patients with a response to treatment who had an increase in the incremental C-peptide response at six months (P=0.03 by repeated-measures analysis of variance for the comparison with the patients with no response). The I bars represent standard deviations.

of these two groups. Furthermore, even after 18 months, the C-peptide response to the mixed-meal tolerance test was the same as or greater than that at diagnosis in 6 of the 11 antibody-treated patients who had been followed for that long.

Accumulated clinical experience, as well as results from the Diabetes Control and Complications Trial[23] and other studies,[24,25] indicate that there is better metabolic control of type 1 diabetes mellitus in patients in whom some insulin secretion is retained. In the Diabetes Control and Complications Trial, a stimulated C-peptide level of more than 0.2 nmol per liter was associated with improved metabolic control, as reflected in the glycosylated hemoglobin level.[23] It is not surprising, therefore, that the improved insulin secretion was accompanied by an improvement in the glycosylated hemoglobin level and a reduction in the insulin needs of patients treated with monoclonal antibody.

Antibody treatment had a sustained effect on the disease in the absence of continued administration of the monoclonal antibody. The effects of this monoclonal antibody on T cells differ from those of previously tested immunosuppressive agents and may account for the more sustained response. Other immunosuppressive agents, including cyclosporine, azathioprine, and prednisone, work by blocking the effector phases of immune responses by interfering with the production of cytokines, the proliferation of T cells, or both. Preclinical studies by Bluestone and colleagues[26-28] suggested that antibody against CD3 that does not bind to the Fc receptor has selective effects on specific populations of T cells. It kills or causes unresponsiveness in T cells that produce interleukin-2 or interferon-γ (type 1 helper T [Th1] cells), whereas T cells that produce interleukin-10 or interleukin-4 (type 2 helper T [Th2] cells) may be stimulated by the monoclonal antibody.[26-28] This effect is seen only in activated T cells and not in naive T cells. The presence of interleukin-10 and interleukin-5 — but not interferon-γ or interleukin-2 — in serum after monoclonal-antibody treatment is consistent with these observations. Studies involving animal models support the importance of Th1 responses in the pathogenesis of type 1 diabetes mellitus, suggesting a mechanism for the effect of monoclonal-antibody treatment.[4,29-32] Clearly, the drug binds all T cells that express the CD3 molecule. Therefore, the selectivity observed among subpopulations of T cells may relate to quantitative or qualitative differences in response to the signal delivered by the monoclonal antibody. This may be analogous to the differential response to altered-peptide ligands by various subpopulations of T cells.[33,34] Thus, the effect of monoclonal-antibody therapy may be to shift the autoimmune response toward production of protective (Th2) cytokines. The rash that developed in most patients, with histologic features similar to those of eczemoid lesions, might be mediated by Th2 responses.[35]

Subjects who had a response to the monoclonal-antibody treatment had an increase in the number of CD8+ T cells after treatment. Several reports have described subpopulations of CD8+ cells in rodents and humans that have immune-regulatory properties.[36-38] Studies are under way to find cell-surface markers that can identify cells associated with a response to monoclonal-antibody treatment and that may indicate the presence of regulatory populations after such treatment.

We did not observe any changes in the titer or the isotypes of anti-GAD autoantibodies. It is possible that these autoantibody responses had already matured at the time of diagnosis and thus were not susceptible to change by circulating cytokines. Similarly, we failed to find an effect of monoclonal-antibody treatment on antirubella IgG titers (mean ratio of patient titers to standard titers at entry, 1.33 ± 0.62; mean ratio at six months, 1.38 ± 0.06), suggesting that established humoral responses were unaffected. Other immunologic markers, including HLA type and the titers and isotypes of autoantibodies, did not predict clinical response. The fasting C-peptide level was higher in the patients who had a response to treatment but was not an absolute predictor of a clinical response to the monoclonal antibody, as it was in the case of cyclosporine treatment of new-onset type 1 diabetes.[5]

Thus, treatment within the first six weeks after the onset of type 1 diabetes mellitus with a single course of anti-CD3 monoclonal antibody appeared to arrest the deterioration of insulin production in the majority of our 12 patients for at least the first year of disease. The mechanism of antibody action is under investigation, but we speculate that the monoclonal antibody may alter the immunologic response that causes type 1 diabetes mellitus, may induce a population of cells that can influence the disease process, or both.

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formed essentially as described [M. E. Greenberg and E. B. Ziff, *Nature (London)* 311, 433 (1984)], but with modifications described elsewhere (*19*). Each nitrocellulose filter "slot" contained 1.0 μg of denatured, immobilized DNA. After hybridization with a solution containing 10^7 count/min per milliliter of RNA sample, filters were washed and exposed to x-ray film for 13 days at −70°C with intensifying screen, and results quantitated by densitometry. The observation of a considerably more intense signal with a ribosomal RNA probe than with probes for mRNA transcripts (Fig. 3) demonstrates that for the latter, immobilized DNA was in excess.

15. The 5′-deletion mutants of pPRL-CAT and pGH-CAT were constructed as follows. To generate pPRL-CAT deletions containing 958 and 618 nucleotides, respectively, of prolactin 5′-flanking sequences, pPRL-CAT was digested with Sma I and then Sph I or Sst I, then treated to make the ends blunt and religated. Further 5′ deletions were prepared by Bal 31 digestion initiated in the polylinker of the −618 deletion. All deletions share the same vector sequences 5′ to the prolactin deletion end point. Deletion end points were determined precisely by dideoxy sequencing [E. Y. Chen and P. J. Seeburg, *DNA* 4, 165 (1985)]. Plasmid pAGH-CAT, with 0.236 kb of growth hormone–gene 5′-flanking sequence, contains the pGH-CAT Bgl II–Bam HI fragment cloned into the pUC 9 Bam HI site.
16. CAT activity was assayed essentially as described [C. M. Gorman, L. F. Moffat, B. H. Howard, *Mol. Cell. Biol.* 2, 1044 (1982)], except that extracts were incubated at 65°C for 5 minutes to inactivate an endogenous deacetylase [M. Mercola, J. Goverman, C. Mirell, K. Calame, *Science* 227, 266 (1985)]. Incubation was at 37°C for 8 to 12 hours, in the linear range of the assay. Increased levels of CAT enzymatic activity were detectable as early as 12 hours after cell fusion.
17. The following results imply that activation also does not require the 38 bp of prolactin gene body sequences present in these pPRL-CAT derivatives. Plasmid pPAmCAT contains the prolactin DNA sequences between Pst I sites at −1957 and −11, linked through a Hind III linker at −11 to the Hind III site of the Hind III–Bam HI CAT gene fragment, cloned into pUC 9. In fused heterocultures, equal levels (*11*) of CAT mRNA were yielded by pPAmCAT and pPRL-CAT.
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Three Recessive Loci Required for Insulin-Dependent Diabetes in Nonobese Diabetic Mice

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A polygenic basis for susceptibility to insulin-dependent diabetes in nonobese diabetic (NOD) mice has been established by outcross to a related inbred strain, nonobese normal (NON). Analysis of first and second backcross progeny has shown that at least three recessive genes are required for development of overt diabetes. One, *Idd-1*[1], is tightly linked to the *H-2K* locus on chromosome 17; another, *Idd-2*[2], is localized proximal to the *Thy-1/Alp-1* cluster on chromosome 9. Segregation of a third, *Idd-3*[3], could be shown in a second backcross. Neither *Idd-1*[1] nor *Idd-2*[2] could individually be identified as the locus controlling insulitis; leukocytic infiltrates in pancreas were common in most asymptomatic BC1 mice. Both F1 and BC1 mice exhibited the unusually high percentage of splenic T lymphocytes characteristic of NOD, suggesting dominant inheritance of this trait. The polygenic control of diabetogenesis in NOD mice, in which a recessive gene linked to the major histocompatibility complex is but one of several controlling loci, suggests that similar polygenic interactions underlie this type of diabetes in humans.

NONOBESE DIABETIC (NOD) MICE derived by Makino *et al.* from the non-inbred ICR strain are a model for insulin-dependent diabetes (Idd) in man (*1*). Both cellular and humoral autoimmunity against pancreatic β cells appears to be a central feature in pathogenesis (*2, 3*). Insulitis, a leukocytic infiltration of the pancreatic islets, is a salient histopathological lesion (*1, 4*). Insulitis was found at approximately 40% frequency in reciprocal [(NOD × NON)F1 × NOD] backcross mice at 9 weeks of age, suggesting control by a single recessive trait inherited from NOD mice (*4*). Subsequently, a diabetogenic recessive gene was associated with the unique NOD *H-2* haplotype on chromosome 17 (Chr 17) when an outcross/backcross analysis was performed between NOD and C3H mice (*5*). However, the low frequency of overt diabetes obtained in this analysis suggested the involvement of more than one NOD-derived recessive gene.

Breeding stock of the NOD strain and a related diabetes-resistant strain, nonobese normal (NON) were used at inbred generation F32 and F35, respectively. We have continued inbreeding and designate our sublines as NOD/Lt and NON/Lt; these inbred strains differ at numerous loci including the major histocompatibility complex (MHC), *H-2* (*4–6*). Consistent with the previously observed diabetes incidence of 80% in females and 10% in males (*1*), NOD/Lt females exhibit a diabetes incidence of >90% by 10 months of age; however, NOD/Lt males exhibit a higher than reported diabetes incidence, averaging 50 to 70% by 10 months when fed diet formulation 96W (Emory Morse Co., Guilford, Connecticut). F1 mice (23 males and 24 females) from reciprocal NOD/Lt × NON/Lt outcrosses were studied for development of hyperglycemia over a 12-month period. In contrast to the high incidence of hyperglycemia in parental NOD/Lt mice of both sexes by 12 months of age, F1 mice of both sexes were uniformly diabetes-resistant. NON/Lt can be distinguished from NOD/Lt mice by the number and functions of T lymphocytes in peripheral blood and spleen, as well as by their *Thy-1* phenotypes [Thy-1.1 and -1.2, respectively (*6*)]. NON/Lt mice develop T-lymphocytopenia associated with an age-dependent decline in responsiveness to concanavalin A whereas NOD mice show a persistent T cell hyperplasia associated with strong T cell mitogenic responses (*7*). However, the numbers and mitogen responsiveness of T lymphocytes in all F1 mice examined were NOD-like (*7*). Indeed, although pancreatic islet structure was intact and numbers of granulated β cells were normal, focal pancreatic lymphocytic infiltrates in perivascular and periductular areas, often abutting islets at one pole (but quite distinct from the insulitis observed in NOD parental mice), were noted in five of nine F1 males and nine of ten females examined histologically at 12 months. Thus, both recessive and dominant traits inherited from NOD were associated with pathogenesis.

In the present study, the genetic polymorphisms distinguishing NOD/Lt from

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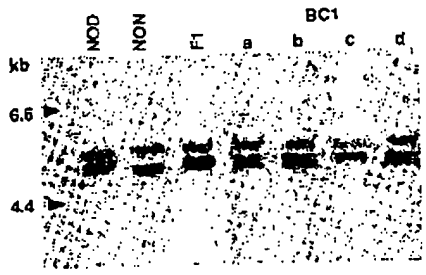

Fig. 1. Southern blot of genomic, Pvu II–digested liver DNA showing the polymorphism distinguishing NOD, NON, and F1 mice at the *P450-3* locus on Chr 9. Two bands are observed; the upper 5.0-kb fragment is common to both strains whereas the anodal lower band is polymorphic (4.8-kb in NOD and 4.6-kb in NON). Lanes a to d show representative RFLP patterns observed in diabetic BC1 mice with the following genotypes: lane a, *Thy-1*^{b}*/Alp-1*^{b}*/Mod-1*^{ab}; lane b, *Thy-1*^{ab}*/Alp-1*^{ab}*/Mod-1*^{ab}; lane c, *Thy-1*^{b}*/Alp-1*^{b}*/Mod-1*^{b}; and lane d, *Thy-1*^{ab}*/Alp-1*^{ab}*/Mod-1*^{b}. These segregation data collectively indicate that *Idd-2* must be located proximal to the *Thy-1*/*Alp-1* markers.

NON/Lt mice on Chr 1, 3, 5, 9, 15, and 17 were utilized to determine the number and the chromosomal localization of the NOD recessive diabetogenic genes. In addition to the reported polymorphic differences between the NOD/Lt and NON/Lt (*6*), we found a restriction fragment length polymorphism (RFLP) at the *P450-3* locus on Chr 9 with Pvu II–digested genomic DNA probed with the full-length complementary DNA clone pP$_3$450FL (*8*). The polymorphic Pvu II fragment in NOD mice is 4.8 kb and in NON mice is 4.6 kb (Fig. 1).

A total of 200 first backcross (BC1) mice were produced from reciprocal backcrosses of F1 mice to the diabetes-susceptible NOD/Lt parental strain. Three mice died of causes other than diabetes and three more died between 8 and 10 months of undiagnosed causes. Only 19 of the BC1 mice (6 males, 13 females) developed overt diabetes (glycemic changes) during the 12-month observation period. This diabetes incidence (9.5%) contrasts sharply with an expected 50% diabetes incidence (uncorrected for penetrance) if a single recessive MHC-linked gene controlling insulitis were segregating and is consistent with a ratio expected for at least three unlinked autosomal recessive genes. All 19 diabetic mice were homozygous for the *H-2K*^{d} allele of NOD; 18 were *I-E*^{o} like NOD (Table 1). The one exception, a *K*^{d}*I-E*^{k} recombinant female, was the first BC1 diabetic to be detected. The classification of this female by MHC-typing of peripheral blood and splenic leukocytes was independently confirmed by RFLP analysis of Bam HI–digested genomic DNA hybridized with an E_β specific probe (*9*). The finding that diabetogenesis was dependent upon homozygosity for the NOD *H-2K*^{d} marker allele confirmed the observation of Hattori *et al.* (*5*) that a diabetogenic recessive trait is MHC-linked. Our data establish that this locus [provisionally named *Idd-1*, in accordance with the rules recommended by The Committee on Standardized Genetic Nomenclature for Mice (*10*)] is tightly linked to the *H-2K* end of the MHC. NOD mice possess the susceptibility allele, *Idd-1*^{s}, and NON a resistance allele, *Idd-1*^{r}. Our evidence does not allow determination of whether the diabetogenic recessive gene is within the MHC, or between the centromere and the *K* end of the *H-2* complex. Since the first mouse to develop diabetes was a recombinant between *H-2K* and *I-E*, the diabetogenic recessive locus would have to be between these two markers if it were within the MHC. The likely position for this recessive gene would be the unique *I-A*$_\beta$ region of NOD (*11*). However, *I-A* genes in heterozygotes are expressed codominantly. Such codominant expression might in part explain why all F1 and BC1 mice analyzed in the present study had the high percentages of splenic T cells characteristic of the NOD strain (Table 2).

Screening the BC1 mice for other polymorphic genetic markers distinguishing NOD/Lt from NON/Lt at Chr 1 *(Idh-1)*, Chr 3 *(Car-2, Amy-1)*, Chr 5 *(Dao-1)*, Chr 9 *(Thy-1, Alp-1, P450-3, Mod-1)*, and Chr 15 *(Gpt-1)* indicated a second recessive diabetogenic gene on the proximal end of Chr 9 located within the 25-centiMorgan (cM) segment between the centromere and *Alp-1* (Table 1). This locus and its respective alleles are provisionally designated *Idd-2*^{s} in the NOD strain, and *Idd-2*^{r} in the NON strain. Thirteen of the diabetic mice shown in Table 1 (including two heterozygotes for *Alp-1*) were also typed for *Thy-1* located 1 cM proximal to *Alp-1*. Since no recombination was observed between these two tightly linked markers (all mice with the NOD b allele at *Alp-1* had the NOD *Thy-1*^{b} allele as well), the *Idd-2* locus is tentatively positioned proximal to the *Thy-1*.

The NON/Lt strain could be differentiated from the NOD/Lt strain in regard to the complete absence of insulitis in the former strain, even after 2 years of age. In NON/Lt mice at 1 year of age and older, focal mononuclear cell infiltrates were observed in association with ducts, blood vessels, and acini, and occasionally, at one pole of an islet. However, insulitis (mononuclear cell infiltration into the islet capsule, with associated islet structural erosion), was not observed in this strain. Histopathologic analysis of the pancreases of nondiabetic BC1 mice surviving to 1 year of age showed a high frequency of leukocytic infiltrates that were completely independent of whether the mice typed homozygous or heterozygous for NOD markers for either or both the *Idd-1*^{s} and *Idd-2*^{s} alleles. Sections of Bouin's-fixed, paraffin-embedded pancreases from each mouse, sampled at three different levels 1000 μm apart, were screened for lymphocytic infiltration; staining with aldehyde fuchsin to detect granulated β cells allowed determination of β cell cytopathology associated with insulitis. Approximately 25 to 35 islets per mouse were assessed. Pancreases of only 17% of asympromatic BC1 mice were free of islet-associated lesions and these included all possible (inferred) *Idd* genotypes (Table 3). Focal leukocytic infiltrates were observed in 83% of the asymp-

Table 1. Genetic profile of diabetic BC1 mice showing localization of two diabetogenic recessive genes on NOD Chr 9 and 17. Phenotypes of apolipoprotein-1 (*Alp-1*) and supernatant malic enzyme (*Mod-1*) were determined on Titan III cellulose acetate plates (Helena Laboratories, Inc.) as described (*15*). Allele a designates a fast anodal electrophoretic variant, allele b a slow variant of the respective protein. RFLP alleles at *P450-3* were determined by Southern blot analysis of 10 μg of liver DNA digested with Pvu II and probed with nick-translated pP$_3$450FL plasmid as described (*8*). D, 4.8-kb fragment in NOD; N, 4.6-kb fragment in NON; DN, both fragments in F1. The MHC haplotype of NOD is *K*^{d} *I-A*$^{?}$ *I-E*^{o} *Sl*^{h} *Slp*^{o} *D*^{b} and of NON is *K*^{b} *I-A*^{o} *I-E*^{k} *Sl*^{h}*Slp*^{o} *D*^{b}. The *I-A* of NOD is unique (*11*) and a haplotype designation is pending; the *I-A* of NON is as yet uncharacterized. *H-2K* and *I-E* were typed in a microcytotoxicity assay (*14*) by means of monoclonal antibodies against *K*^{d} (31-3-4), *K*^{b} (28-13-3S), and *E*^{k} (17-3-3) determinants. All BC1 mice were typed; this table shows the genotypes of the 19 mice that developed diabetes.

Strain	Chromosome 9			Chromosome 17		Number observed
	Alp-1	*P450-3*	*Mod-1*	*H-2K*	*I-E*	
NOD	b	D	b	d	o	
NON	a	N	a	b	k	
F1	ab	DN	ab	db	k	
BC1	b	D	b	d	o	11
	b	D	ab	d	o	1
	b	DN	ab	d	o	3
	ab	DN	b	d	o	2
	ab	DN	ab	d	o	1
	b	D	ab	d	k	1

Table 2. Inheritance of T-lymphocyte hyperplasia.

Strain	Phenotype		Percent viable splenic leukocytes (n)*		Age range (weeks)
	H-2K	*Thy-1*	T cells	B cells	
NOD	dd	.2/.2	42.6 ± 1.95 (5)	53.2 ± 1.12 (5)	30
NON	bb	.1/.1	15.1 ± 2.52 (8)	57.2 ± 2.51 (8)	20
F1	bd	.1/.2	39.5 ± 2.37 (4)	46.6 ± 2.27 (4)	26
BC1	dd	.2/.2	37.9 ± 1.99 (31)	42.4 ± 1.84 (31)	14–44
BC1	dd	.1/.2	33.3 ± 1.56 (7)	48.2 ± 2.67 (7)	12–42
BC1	db	.2/.2	35.4 ± 2.21 (13)	50.8 ± 2.39 (13)	36–44
BC1	db	.1/.2	29.0 ± 2.16 (11)	52.1 ± 2.96 (11)	36–44

*Values represent the mean percentage ± SEM of viable splenic leukocytes for the indicated number of mice (n). Separate aliquots of 2 × 10^6 splenocytes were incubated with or without mouse monoclonal antibody to Thy-1.1 (anti–Thy-1.1) or anti–Thy-1.2 for 30 minutes, washed, and then stained for an additional 30 minutes with fluoresceinated goat anti-mouse immunoglobulin, prior to enumeration in an Ortho cytofluorograph. T lymphocyte levels were calculated by subtracting the percentage of splenocytes staining positive for surface immunoglobulin in the absence of anti–Thy-1 from those incubated with anti–Thy-1 followed by anti-mouse immunoglobulin. T cell percentages of F1 and all BC1 genotypes were significantly different from NON ($P \leq 0.001$), but F1 percentages were not significantly different from NOD. Both groups of BC1 mice heterozygous at *Thy-1* had a significantly lower percentage in comparison to NOD ($P \leq 0.003$).

tomatic BC1 mice of both sexes. Although primarily concentrated peripherally to structurally intact islets (periductular and perivascular as observed in F1 mice), lymphocytic penetration into variable numbers of islets (insulitis) was clearly observed in 27% of the pancreases of asymptomatic mice, again without correlation to (inferred) homozygosity for either or both of the *Idd-1*^{s} and *Idd-2*^{s} alleles. Although insulitis was scored in only a few of the islets most of the islets with or without the periinsular leukocytic aggregations were structurally intact and filled with well-granulated β cells, thus reflecting the normoglycemic status of the mice.

If only two diabetogenic recessive genes inherited from the NOD strain are required for diabetogenesis, then a maximum of 25% of BC1 mice should have developed diabetes (assuming full penetrance). On the contrary, if three recessive *Idd* alleles were required, a theoretical maximum incidence of 12.5% would be expected at BC1 (very close to the 9.5% observed). We tested the hypothesis that the 27% (37/139) of nondiabetic BC1 mice that were homozygous for NOD-type marker alleles on Chr 9 and 17 (and therefore presumably also for *Idd-1*^{s} and *Idd-2*^{s}) remained diabetes-free due to heterozygosity at a third recessive locus *(Idd-3)*. Nondiabetic BC1 males homozygous for NOD Chr 9 and 17 markers were selected for a second backcross (BC2) to NOD females. If these selected BC1 breeders were diabetes-resistant because of heterozygosity at a third locus (*Idd-3*^{s}/*Idd-3*^{r}), then the maximum incidence of overt diabetes in the BC2 generation would theoretically be 50% (assuming complete penetrance). A diabetes incidence of 46% (27 of 59 mice, 12 males and 15 females) was obtained by 10 months of age; this strongly supported the conclusion that NOD mice differ from NON mice by

Table 3. Dissociation of pancreatic leukocytic infiltrates from *Idd-1*^{s} and *Idd-2*^{s} diabetogenic alleles inherited from NOD. Data are from 139 aglycosuric and normoglycemic BC1 mice surviving to 1 year of age. Phenotypes for *Alp-1* and *Mod-1* markers are described in Table 1.

Marker genes			Linked diabetogenic genes*		Number of mice with degree of leukocytic infiltration†				Total
Alp-1	*Mod-1*	*H-2K*	*Idd-1*	*Idd-2*	0	1	2	1 and 2	
b	b	d	s	s	2	5	22	8	37
b	ab	d	s	s	1	1	3	1	6
ab	ab	db	sr	sr	3	2	14	1	20
ab	b	db	sr	sr	0	0	5	0	5
b	b	db	sr	s	11	0	16	8	35
ab	ab	d	s	sr	5	2	11	3	21
ab	b	d	s	sr	0	2	2	1	5
b	ab	db	sr	s	1	1	6	2	10
				Total	23	13	79	24	139

*s = NOD susceptibility allele; r = NON resistance allele. Since none of these mice were diabetic, the genotypes at *Idd-1* and *Idd-2* are inferred on the basis of marker genes. †Intensity of leukocytic infiltrates are scored as follows: 0 = none, with no visible lesions to islets; 1 = insulitis (penetration of lymphocytes into the islet capsule, with islet cells exhibiting cytoarchitectural disarray and erosion of beta cell mass); 2 = periductular and perivascular lymphocytic aggregations, often localized at one pole or surrounding islets which are completely normal in structure and aldehyde fuchsin staining properties.

susceptibility alleles at only the one a
tional locus. No linkage of *Idd-3*^{s} to in
mative biochemical genetic markers sc
gating in BC2 at Chr 1, 3, 5, or 15 coul
shown.

Our results indicate that the genetic b
of diabetes susceptibility in the NOI
strain versus resistance in the NON/Lt st
is attributable to polygenic interactions c
least three NOD recessive genes and pc
bly also of (co)dominant determinants
herited from the NOD/Lt strain that con
T cell hyperplasia. Makino *et al.* (*4*) scree
pancreases of 9-week-old prediabetic (N
× NOD)F2 and BC1 mice for insul
their data suggested that genetic contro
diabetogenesis in these closely related str
was mediated by a single autosomal lc
producing presence or absence of insul
Given the widespread distribution
leukocytic infiltrates in pancreases of 1-y
old (NON × NOD)F1 and BC1 n
found in the present study, it seems unli
that *Idd-1*^{s}, *Idd-2*^{s}, or *Idd-3*^{s} individu
represents the recessive locus controlling
presence of insulitis. The pancreatic agg
gations may reflect the dominant T
lymphoproliferative drive inherited fr
NOD. In F1 and nondiabetic BC1 indiv
uals, these aggregates were predominar
localized at the islet capsule perimeters ra
er than infiltrating into the core of the is
(insulitis).

Our study indicates that a complex pc
genic interaction is required for elicit
sufficient β-cell destruction to be reflec
by widespread insulitis and developmen
overt diabetes. This study has only elucic
ed diabetogenic genes that distingu
NOD/Lt from NON/Lt mice. That th
closely related strains share numerous dia
togenic susceptibility loci was clearly ill
trated when outcross/backcross analysis v
performed between NOD and an unrela
strain, C57BL/KsJ, selected because of
well-established sensitivity to diabetoge
chemicals and obesity genes (*7*). Only
BC1 female of 115 mice (0.9%) produ
by backcross of (C57BL/KsJ × NOD/Lt)
to NOD/Lt mice developed diabetes, in
cating that NOD/Lt and C57BL/KsJ m
differ at a minimum of six diabetoge
recessive loci.

This polygenic basis of insulin-dependi
diabetes inheritance in mice is similar to d
in rats (*12*) and clearly indicates that analy
of a susceptibility genotype for diabe
mellitus in humans should not be limi
only to analysis of HLA-linked genes. Lo
tion of non-MHC–linked diabetoge
genes in NOD mice should prove valua
in suggesting other human chromoson
that may carry loci affecting autoimmun
against pancreatic β cells. In humans,

THY-1 and *ALP-1* loci are found on the long arm of Chr 11; it is therefore reasonable to suggest this chromosome as a potential site for a diabetogenic locus analogous to *Idd-2*.

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Modern Turtle Origins: The Oldest Known Cryptodire

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The discovery of a turtle in the Early Jurassic (185 million years before present) Kayenta Formation of northeastern Arizona provides significant evidence about the origin of modern turtles. This new taxon possesses many of the primitive features expected in the hypothetical common ancestor of pleurodires and cryptodires, the two groups of modern turtles. It is identified as the oldest known cryptodire because of the presence of a distinctive cryptodiran jaw mechanism consisting of a trochlea over the otic chamber that redirects the line of action of the adductor muscle. Aquatic habits appear to have developed very early in turtle evolution. *Kayentachelys* extends the known record of cryptodires back at least 45 million years and documents a very early stage in the evolution of modern turtles.

THE EVOLUTIONARY HISTORY OF turtles extends at least to Late Triassic time (200 million years before present), but the fossil record of their early diversification is incomplete. A substantial structural and temporal hiatus exists between the most primitive known form, *Proganochelys quenstedti* of the Late Triassic (middle Keuper of Germany) (*1*), and turtles with essentially modern features, which first appeared in the Late Jurassic (140 million years before present) (*2*). *Proganochelys* has a shell and is hypothesized as the sister group of all other turtles (*3*), but the skull has relatively few chelonian features. There are no characters of *Proganochelys* that indicate that this genus belongs to either the cryptodires or the pleurodires, the two groups of modern turtles. Both cryptodires and pleurodires possess distinctive specializations of the skull and posteranial skeleton consistent with the interpretation that they are monophyletic, and together they are united in a single monophyletic taxon, the Casichelydia (*4*, *5*). The discovery of an Early Jurassic (185 million years before present) cryptodire (Fig. 1) extends the known history of the cryptodires back more than 45 million years and documents an intermediate stage in the evolution of modern turtles; the skull and shell structure of this new form represents an appropriate ancestral morphotype for all Cryptodira.

The systematics:

Order Testudines
  Gigaorder Casichelydia
    Megaorder Cryptodira
      Family Kayentachelyidae, new
        Kayentachelys, new genus

Type species: *Kayentachelys aprix*, new species.

Known distribution: Early Jurassic, Arizona, United States.

Etymology: Kayenta, for the Kayenta Formation; *chelys*, Greek for turtle.

Diagnosis: As for species.

Kayentachelys aprix, new species

Type specimen: Museum of Northern Arizona V1558.

Locality: Gold Spring, Adeii Eechii Cliffs, Coconino County, Arizona (35°45′35″N, 111°04′51″W).

Horizon: Silty facies of the Kayenta Formation, Early Jurassic.

Etymology: *aprix*, Greek for tight, in reference to the fused basicranial articulation.

Referred specimens: MNA V1559-V1570; V2664. MCZ 8914-8917.

Diagnosis: A combination of primitive and advanced characters (*6*). Primitive amniote characters: pterygoid teeth, interpterygoid vacuity; prootic exposed ventrally. Primitive chelonian characters: nine costals; epiplastron with dorsal process. Derived casichelydian characters: antrum postoticum; fused basipterygoid articulation; 11 peripheral bones. Derived cryptodiran characters: processus trochlearis oticum; processes pterygoideus externus projecting posteriorly with a flat, vertical plate (*7*).

Turtles have often been cited as examples of "living fossils," a group that is structurally conservative throughout its history. In fact, however, this viewpoint is erroneous. Although all turtles appear superficially similar because they have a shell, there have been fundamental cranial and posteranial changes during their history. Cryptodires and pleurodires (Fig. 2), the two groups of living turtles, have independently evolved different trochlear mechanisms that redirect the main tendon of the jaw adductor muscle (*8*). As a result, the adductor, which has expanded posteriorly relative to the jaw joint, maintains a vertical line of action during jaw closure. In cryptodires the trochlea is formed by a thickened protuberance on the anterodorsal portion of the prootic and quadrate, whereas in pleurodires the trochlea is a lateral process of the pterygoid. *Kayentachelys* has the cryptodire trochlear condition. Another characteristic of cryptodires is an extensive fusion of the palatoquadrate and neurocranium that encloses the middle ear. Primitive amniotes and *Proganochelys* lack these advanced characters of the ear region and also have an open basipterygoid articulation. *Kayentachelys* represents an intermediate stage in that it has a fused basipterygoid articulation but lacks the distinctive cryptodiran posteroventral floor of the middle ear, exposing the prootic in ventral view. *Kayentachelys* also retains features that are primitive for turtles such as palatal teeth, an interpterygoid vacuity, a dorsal process on the epiplastron, and a ninth costal bone in the carapace. *Kayentachelys* thus shows that cryptodires evolved their distinctive trochlear pattern early in

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